VIGOR DETERIORATION IN SOYBEAN SEEDS (*Glycine max* (L.) Merr.) AFTER ACCELERATED AGING

BY

DAVID FLOREN JAMES

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF THE
UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1982

MMI 89

To all who helped.

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. Daniel J. Cantliffe, chairman of the supervisory committee. His support, guidance, and friendship provided throughout this study will always be highly valued.

The advice and assistance of the other supervisory committee members, Dr. C. B. Hall, Dr. T. E. Humpheys, Dr. T. A. Nell, and Dr. S. H. West, are gratefully acknowledged. The author would also like to acknowledge the Herman Frasch Foundation for their interest and support of the research work.

Special thanks are offered to Jeanne Fischer and Debi Gaw for their technical assistance and to Linda Drake for her expert typing of this manuscript.

The friendship and help of Roger Styer, Jim Watkins, Charlie Cottingham, and Tom Peeler are very much appreciated

The author would like to sincerely thank his wife, Holly, for her love, assistance, and understanding.

Above all, deepest gratitude is extended to the author's parents, for without their help and encouragement this study would not have been possible. Their unselfishness will not be forgotten.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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May, 1982

Chairman: Daniel J. Cantliffe
Major Department: Vegetable Crops

Several factors related to metabolic energy production and utilization were investigated as to their role and importance in soybean seed vigor deterioration after accelerated aging.

During a 96 hour germination period aged seeds exhibited slower axis fresh and dry weight accumulation and shorter axis lengths, but there was no loss in germinability compared to nonaged seeds. A leachate test indicated that the aging stress led to greater electrolyte leakage. The percent of solute loss was much higher in axes than in intact seeds.

Respiration of aged seed axes was impaired during the first 24 hours of imbibition but exceeded that of nonaged seeds during the following 72 hours of seedling growth. Neither energy charge nor the concentrations of ATP or ADP in the axes of intact seeds or seedlings reflected vigor deterioration. However, the concentrations of AMP and total phosphorylated adenylates were generally higher in the axes of nonaged seedlings compared to aged seedlings.

Accelerated aging had no effect on the initial amounts of total soluble sugars or reducing sugars in the seed. During germination, reducing sugars comprised a smaller proportion of the total soluble sugars in the aged axes than in the nonaged axes until axis dry weight accumulation became evident at 72 hours. A greater quantity of amino acids was detected in the cotyledons of unimbibed seeds after accelerated aging suggesting degradation of stored reserve proteins. An increase in amino acid content was noted in nonaged axes 48 hours after imbibition but was delayed 24 hours in aged axes.

Axis protein synthesis was impaired in aged seeds throughout the first 72 hours of germination but eventually reached a peak in activity comparable to nonaged seeds. Aging not only delayed axis root tip cell division but reduced its activity relative to nonaged seeds.

External sucrose concentrations up to 5% improved the growth of excised nonaged axes but had no effect on the growth of excised aged axes, although respiration was stimulated. Energy charge evaluations and ATP concentrations generally appeared to be unrelated to differences in excised axis growth associated with vigor deterioration.

INTRODUCTION

High temperature and relative humidity can hasten the rate of aging and hence the extent of vigor deterioration of soybeans and many other types of seeds. These adverse environmental factors are most commonly associated with improper storage conditions but can also be imposed by delayed harvesting of the matured seeds or during their transport and handling. Therefore, the vulnerability of seeds to high temperature and relative humidity stress is a problem in both the production and maintenance of high quality seeds.

This knowledge has led to the development of stress testing seeds by accelerated aging, whereby vigor deterioration is rapidly induced by subjecting seeds to supraoptimal temperature and relative humidity for a short period of time. The technique has proven useful for estimating the vigor of seed lots in terms of potential storability as well as field performance (Bishnoi and Delouche, 1980; Byrd and Delouche, 1971; Caldwell, 1960; Roos and Manalo, 1971; TeKrony, 1973). A basic assumption has been that vigor deterioration resulting from accelerated aging is similar to that which occurs as seeds age under conventional storage conditions.

Vigor deterioration may be expressed in several ways prior to the death of the seed. These deleterious effects include a reduction in the rate and uniformity of germination and seedling growth and an increase in abnormal seedling development. From a practical standpoint, planting of deteriorated seeds, particularly under unfavorable field environments,

may lead to poor emergence, inadequate plant stands, and decreased crop yields.

While the morphological manifestations of vigor deterioration may be readily discernable, the physiological and biochemical mechanisms that detract from or contribute to optimum seedling growth have not been fully characterized. Reduced vigor of aged seeds has been partially attributed to the impairment of several metabolic processes identified prior to normal seedling development, and partially due to the physical and/or biochemical alteration of membranes. However, little is known about the role or activity of these processes through the period of early seedling development. Furthermore, minimal attention has been focused on the relationship of seed vigor expression to changes in the levels of stored reserve materials and biochemical intermediates during germination and early seedling growth. Therefore, studies involving soybean seed vigor deterioration induced by accelerated aging were undertaken with the following objectives: (1) to investigate seed and axis membrane permeability in relation to the retention of stored reserve materials upon imbibition, (2) to examine several aspects of energy production and utilization during germination and early seedling growth, and (3) to determine the effect of external carbohydrate availability on excised axis growth, respiratory activity, and phosphorylated adenylate content and energy charge.

In addition to providing a more thorough understanding of the biochemical basis of soybean seed vigor deterioration, these studies may aid in improving methods of maintaining and biochemically assessing seed vigor. The results may also be of benefit to developing techniques for enhancing the vigor of deteriorated seeds.

CHAPTER I
LITERATURE REVIEW

Terminology and Concepts of Seed Vigor and Aging

Several definitions can be found in the scientific literature to define seed vigor. Most of these are based on one or more properties associated with seed germination, early seedling growth, and in certain instances, yield of a marketable product.

The German word Triebkraft, originated in the early 1900's, meaning both "shoot strength" and "driving force," is reported to be the earliest reference to the concept of seed vigor (Heydecker, 1972, p. 209). The word was used to explain the observation that cereal seeds infected with Fusarium spp. germinated but were unable to emerge through a layer of crushed brick under monitored environmental conditions.

Isley (1957, p. 181) proposed a more formal definition, stating seed vigor as "the sum total of all seed attributes which favor stand establishment under unfavorable field conditions." This interpretation implies that relative levels of vigor are only evident when germination takes place under suboptimum conditions. Delouche and Caldwell (1960, p. 125) amended the definition to include the germination performance of seeds under favorable as well as adverse environments. They stated vigor as "the sum total of all seed attributes which favor rapid and uniform stand establishment in the field."

Copeland (1976) suggested that vigor should take into account the following factors related to seedling growth: speed of germination,

uniformity of germination and plant development under non-uniform conditions, the ability to emerge through crusted soil, germination and seedling emergence from cold wet pathogen infected soil, normal seedling morphological development, crop yield, and storability under a wide range of conditions.

On an individual seed basis Abdul-Baki (1980, p. 765) defined vigor as "the capacity of a single seed to produce one plant of marketable product." He added that "vigor as it applies to a population of seeds, is the ability to maintain rapid, uniform, and high emergence under the broadest range of environmental conditions."

To date, no official definition of seed vigor has been adopted by the Association of Official Seed Analysts. A draft version has been proposed stating "seed vigor is the sum total of all those properties in seeds which, upon planting, result in rapid uniform production of healthy seedlings under a wide range of environments including both favorable and stress conditions" (Woodstock, 1976, p. 4). The International Seed Testing Association has also proposed a definition of seed vigor. Unofficially, they defined vigor as "the sum total of those properties of the seed which determine the potential level of performance and activity of a nondormant seed or seed lot during germination and seedling emergence" (Woodstock, 1976, p. 6). Implied in both definitions is the idea that a seed must germinate before vigor can be expressed. However, the latter definition distinguishes vigor as not being a function of seed dormancy.

An incomplete identification of factors influencing germination and seedling growth has perhaps been partially responsible for the absence of a universally accepted definition of seed vigor. According to the

author of this review, vigor may be considered as the capacity of a seed lot to germinate rapidly and uniformly and exhibit optimum seedling growth over the broadest range of environmental conditions.

One important factor governing germination, seedling growth, and ultimately vigor, is seed aging. In a general sense, aging is a biological phenomenon common to all living organisms with the theoretical exception of some single-celled organisms such as bacteria (Strehler, 1977). Despite the familiarity and universal nature of the word, the precise meaning of aging in a scientific context is not always clear. This confusion over terminology is evidenced by the various definitions and interpretations of aging.

Leopold (1980, p. 3) has referred to aging as "processes of accruing maturity with the passage of time." Other individuals have considered aging to occur only after physiological maturity has been reached (Harrington, 1972; Lamb, 1977).

In a negative sense, Comfort (1960, p. 4) described aging as "an increased liability to die, or an increasing loss of vigor, with increasing chronological age, or with the passage of the life cycle." Wareing and Seth (1967, p. 543) suggested that aging should be reserved for changes causing a decreased efficiency of an organ or organism, specifically stating "those which are degradative or degenerative in nature." Similarly, aging processes have been defined as "those which render an individual more susceptible as they grow older to various factors, intrinsic or extrinsic, which may cause death" (Smith, 1962, p. 115). However, according to Leopold (1980), aging processes are not all necessarily detrimental, but include other changes having no apparent effect on an organism's survival capacity.

Although conflicting explanations of aging exist, several important points can be made about the major concepts of physiological aging. First, aging proceeds according to a genetically predetermined sequence (Bellamy, 1967), thus accounting for general differences in longevity among various species. Second, the rate of aging is modified by the interaction of an organism with its environment. This aspect of aging is particularly true of organisms having minimal control over their internal temperature and moisture content (Roberts, 1979). Third, the changes in cell processes due to aging are irreversible and therefore cumulative (Anderson, 1973; Lamb, 1977). Lastly, these changes are primarily deleterious, as the final result of aging is death (Lamb, 1977).

Considering these general concepts, physiological seed aging may be explained as the cumulative changes occurring with time in mature seeds which primarily result in deterioration of structure and function, thus reducing vigor and ultimately leading to a loss of viability.

Environmental Factors Regulating Seed Aging

The interaction of environmental conditions with the seed's genetic capacity for vigor plays an important role in regulating the rate of aging. Helmer et al. (1962) and others (Anderson, 1973; Harrington, 1972; Maguire, 1977) have suggested that seeds reach their highest potential germination performance or vigor at physiological maturity. This developmental stage in soybeans and certain other crops is recognized as the point where maximum seed dry weight is attained (Harrington, 1972; TeKrony et al., 1979). Physiological maturity also coincides with the initiation of seed aging (Anderson, 1973). Because seeds are subject to variations in environmental stress, the rate of vigor

deterioration due to aging cannot be based exclusively on chronological time (Roberts, 1979). In addition to time, the 2 most important environmental factors controlling the rate of aging are temperature and moisture (Harrington, 1972).

Mondragon and Potts (1974) found that aging of physiologically mature soybean seeds was initiated prior to storage on the mother plant under warm humid field conditions. Over a 5 week post-maturation period the germination percentage dropped rapidly. If plants were shaded during this time, a higher degree of germinability was observed. They attributed the slower rate of aging to a more stable microenvironment, specifically the temperature and relative humidity, which surrounded the shaded plants.

After harvest, seeds are rarely planted without undergoing a period of time in storage. During storage, temperature and moisture continue to exert a significant effect on the rate of seed aging. Reduction in viability has perhaps been the most widely used index for measuring age induced deterioration when seed lots have been stored over a range of temperatures and moisture conditions for prolonged times. However, before a loss in viability occurs, adverse environmental conditions may initiate aging processes which decrease the physical characteristics associated with vigorous seedling growth.

As reviewed by Barton (1961) and Owen (1957) numerous experiments with a variety of crop seeds have shown that loss of viability or seedling vigor was hastened as seed moisture and/or temperature was increased during storage. For example, 2 varieties of soybeans were each stored at 3 different moisture contents at a constant 20^{o} C temperature (Toole and Toole, 1946). At 18% moisture, seeds from both

varieties remained viable for only 5 to 9 months, while at 13.5% moisture, viability did not decline for 2 years. Seeds containing 8% moisture had 90% germination after 5 years and lost viability gradually in subsequent years. A reduction in germination more readily occurred in all seeds stored at 38° C. At lower temperatures, seeds of comparable moisture contents retained greater viability for longer periods of time than at 20° C.

Ching et al. (1959) reported similar results with crimson clover and ryegrass stored at 22° C. Seeds at 6% or 8% moisture readily germinated after 3 years, but at 16% and 20% moisture viability was lost by 12 and 6 months, respectively. Furthermore, as storage time increased abnormal seedlings comprised a greater proportion of the seeds that did germinate from the high moisture treatments. The rate of aging was decreased by lowering the temperature to 5° C over the 3 year period. Viability was maintained in the 16% moisture seeds in addition to those at lower moisture levels at this temperature. A considerably slower rate of viability loss was also observed in seeds containing 20% moisture.

Harrison (1977) stored barley seeds with 20% moisture at 20° C. Aging under these conditions resulted in slower germination and seedling emergence and reduced root and shoot growth which was attributed to decreased meristematic activity. Plants from aged seeds started to tiller later than those from nondeteriorated seeds, but tillering rates were similar. Another inferior growth characteristic observed in plants from aged seeds was significantly shorter leaf lengths. However, by harvest there was no apparent difference in grain yields.

In a study by Barton and Garmon (1946) pepper, lettuce, and tomato seeds were stored for as long as 13 years before they evaluated the

effects of aging relative to fresh seeds. All 3 types of seeds were stored at reduced moisture contents in sealed containers. In addition to this storage treatment, lettuce and tomato seeds were kept in open containers at room temperature for the same time period. Differences in vigor deterioration were observed but varied among species. No significant differences in plant or yield could be attributed to the aging of pepper seeds in sealed containers at low temperature, although the germination percentages were lower. Aged lettuce seeds also showed a reduction in germination compared to fresh seeds, but older seeds, regardless of storage conditions produced heads of larger weight than those from fresh seeds. Only when tomato seeds were stored at room temperature in open containers was a reduction in growth and yield observed. Tomato plants from seeds stored in sealed containers at low temperature were similar in every respect (germination capacity, growth, and yield) to plants from fresh seeds. The authors summarized their results by stating that chronological time was of less importance in regard to aging and vigor deterioration than the environmental conditions under which the seeds were stored.

If low moisture seeds are stored under high relative humidity, seed moisture content may increase and thus speed the rate of aging. Illustrating this point, Halder and Gupta (1980) stored sunflower seeds with an initial moisture content of 7% under different humidity conditions. Storage of all lots was at 28^{o} C. After 150 days, seeds held at 95% relative humidity had increased moisture content to a maximum of 16%. Yet, all viability was lost after 90 days. The seeds contained only 13% moisture at that time. A 40% reduction in germination was observed in seeds stored at 85% relative humidity where the seed

moisture content eventually reached 11%. At 50% relative humidity, seed moisture content actually declined to 5% with a 10% reduction in viability after 3 months of storage.

Increasing relative humidity at a constant storage temperature not only resulted in decreased viability with time but also lowered the germination rate and emergence potential of corn seeds from cold moist soil conditions (Gill and Delouche, 1973). Both root and shoot growth decreased with storage time under all environments but was more severe when seeds were exposed to greater relative humidities.

Susceptibility to aging at high seed moisture contents is even more apparent in short-lived seeds when held at conventional storage temperatures. Rocha (1958) noted a loss in viability in onion seeds within 2 weeks after being stored at 15% moisture. Nearly all seeds were dead by 3.5 months. In contrast, seeds at 6.5% moisture maintained their initial germination capacity after 7.5 months.

Nutile (1964) found that extremely low seed moisture could be as detrimental to the rate of seed aging as high seed moisture. Viability, speed of germination, and normal seedling development of several types of seeds decreased with increasing storage time at room temperature when moisture contents were lower than 2%. At a lower temperature (7^{o} C) the rate of aging was reduced as shown by improved germination performance and less seedling injury.

Accelerated Aging

The realization that the rate of seed aging can be hastened during storage if temperature and seed moisture are increased has provided the basis for accelerated aging of seeds. As opposed to a relatively slow rate of aging usually associated with conventional seed storage

conditions, accelerated aging subjects unimbibed seeds to supraoptimal temperature (40 to 45° C) and relative humidity (100%) stress for short periods of time (hours or days instead of months or years) resulting in a potentially rapid rate of vigor deterioration (McDonald, 1975).

Accelerated aging has been a frequently used technique in experimental studies focusing on impairment of biochemical mechanisms accompanying vigor loss after the seed has been harvested. Results of these studies will be reviewed later. In a more applied sense, accelerated aging was originally developed as a method for predicting seed storability (Delouche and Baskin, 1973). Relative to both applications is the basic assumption that the processes of deterioration under accelerated aging conditions are similar to those taking place under more normal ranges of storage parameters, only the rate of aging is enormously increased (Delouche and Baskin, 1973; Woodstock and Tao, 1981). Therefore, differences between ordinary chronological aging and accelerated aging have generally been considered as being a matter of degree rather than kind (Villiers, 1980).

Much of the original work in developing and determining the effectiveness of accelerated aging as a means for evaluating the storage potential of various kinds of seeds was done by Delouche and Baskin (1973). Using a variety of agronomic and vegetable seeds, it was demonstrated that lots having a high survival capacity following accelerated aging would store well under a wide range of environmental conditions. In contrast, seeds lots in which viability was more severely reduced by accelerated aging would not store well. These differences in storability were not evident prior to accelerated aging, since all seed lots had high initial levels of germinability. Similar

conclusions were noted in research conducted by Byrd and Delouche (1971), Egli et al. (1979), and Helmer et al. (1962).

In addition to the use of accelerated aging for evaluating seed storability, further work has demonstrated that the technique may be used to measure vigor in terms of field performance. Helmer et al. (1962) found that the germination of accelerated aged clover seeds closely correlated with soil emergence potential. Similar findings have also been reported for other crops, including cotton (Bishnoi and Delouche, 1980), soybean (Byrd and Delouche, 1971; TeKrony, 1973), pea (Caldwell, 1960), and bean (Roos and Manalo, 1971).

McDonald (1980) has stated that accelerated aging possesses the essential requirements of a seed vigor test: rapid, simple, applicable for most all seeds, capability for individual seed evaluation, and requires no additional training for correct evaluation. However, inconsistant results among seed testing laboratories have not allowed for its acceptance as a standardized vigor test (Tao, 1978). This variability in results has been attributed, in part, to differences in initial moisture of seed lots (McDonald, 1977; Tao, 1979), the type of aging container, sample size, and control of temperature and relative humidity during the aging treatment (McDonald and Phaneendranath, 1978).

Biochemical Characteristics of Aged Seeds

Germination is initiated with the imbibition of water provided adequate environmental conditions exist. The viable seed then moves from a quiescent state to one of progressively greater metabolic activity. The extent to which this transition properly takes place has a direct bearing on subsequent seedling growth. Aging mechanisms appear

to be closely related to impairment of various interrelated biochemical systems in the seed, thus limiting optimum seedling growth.

Membrane Alterations

One of the first events to occur in normally germinating seeds is a physical rearrangement of cellular membranes (Abdul-Baki, 1980). Membranes in dry seeds are thought to be disorganized as cellular contents readily leak from the seed at the start of imbibition, but upon further hydration solute loss decreases substantially (Simon and Raja Harun, 1972). Working with soybean seeds Parrish and Leopold (1977) found that the cotyledons imbibed water rapidly for about the first 10 minutes followed by a slower linear rate of uptake. Solute leakage followed much the same pattern with an initially high rate, declining after 5 to 10 minutes. Simon (1974) has suggested that such observations reflect the conversion of the phospholipid components of membranes from porous hexogonal arrays, present in the dry seed, to intact lamellar layers possessing differential permeability. Supporting this hypothesis of membrane reorganization are ultrastructural studies of dry and imbibed parenchyma cells of soybean cotyledons (Baird et al., 1979) and radicles (Webster and Leopold, 1977). The plasma membrane of cells from dry seeds appeared disrupted and segmented, but after a 20 minute imbibition period formed a continuous barrier enclosing the cytoplasm. Endoplasmic reticulum, not apparent in dry cotyledon tissue, became evident throughout the cytoplasm. Mitochondria initially distorted and devoid of internal structure regained their typical oval shape with numerous cristae and a dense stroma.

In contrast to normally germinating seeds, Ching and Schoolcraft (1968) provided evidence that membrane reestablishment is impaired in

aged seeds stored for extended times. In addition to reduced germination and seedling growth, 10-year-old crimson clover seeds leaked considerably more electrolytes and amino acids than fresh seeds after 30 minutes of imbibition. Parrish and Leopold (1978) noted a similar response from soybean seeds subjected to accelerated aging. Immediate solute loss from imbibing cotyledons increased proportionally with increasing duration of the aging treatment.

Recently, Woodstock and Tao (1981) imbibed excised axes from accelerated aged soybean seeds on blotters containing 30% polyethylene glycol. The treatment not only reduced the rate of water uptake and electrolyte leakage but also resulted in improved axis growth. They concluded that osmotic control of water uptake allowed additional time for membrane rearrangement or repair, thus permitting aged axes to develop in a comparable manner to nonaged axes.

Cytological examinations of corn embryos by Berjak and Villiers (1972a; 1972b; 1972c; 1972d) showed membrane and organelle abnormalities increased with the extent of aging. Cellular damage in all aged seeds was noticeable soon after the start of imbibition. Cells from aged seed embryos which subsequently germinated showed reversal of organelle damage by the time radicles had emerged, suggesting repair mechanisms were operative. On the other hand, organelles in nonviable seeds continued to degenerate indicating membranes in these cells were ineffective in compartmentalizing hydrolytic enzymes. Similarly, Hallam et al. (1973) reported prolonged aging led to loss of mitochondrial membrane integrity in rye embryos which allowed their contents to be dispersed into the cytoplasm upon hydration.

The mechanisms by which membranes may be damaged have not been clearly identified. Several investigators have examined the possibility that injury during aging is the result of free radical formation and the oxidation of unsaturated membrane lipids.

Using cucumber seeds, Koostra and Harrington (1969) were first to report that products of phospholipid oxidation increased with a reduction in viability. Larger decreases in phospholipids and viability were observed from accelerated aged seeds compared to those seeds aged under conventional storage conditions.

When pea seeds were stored under favorable conditions for up to 10 weeks, the level of linolenic acid, and to a lesser degree linoleic acid, declined with increasing age (Harmon and Mattick, 1976). Loss of vigor paralleled the drop in these unsaturated fatty acids, noted in both the axis and cotyledons. Since the concentration of monoenoic and saturated fatty acids was unchanged, the conclusion drawn was that free radical formation rather than beta oxidation was the primary factor involved in the observed responses.

Priestley and Leopold (1979) were unable to detect any indication of lipid oxidation in soybean seeds during 5 days of accelerated aging, while germination and axis growth steadily deteriorated. Phospholipids decreased slightly, but the proportion of saturated to unsaturated fatty acids in the total and polar lipid fractions remained constant. According to the authors, the results suggested that lipid oxidation was not responsible for vigor deterioration induced by accelerated aging.

In contrast to Priestley and Leopold's results with whole seeds, Stewart and Bewley (1980) found that lipid peroxidation may take place

within the axes of aged soybean seeds. During storage at high humidity and temperature the amount of unsaturated fatty acids in the polar lipid fraction decreased and coincided with increases in saturated fatty acids, electrolyte leakage, and the number of nonviable seeds. These observations were not as apparent in axes from aged seeds held under elevated temperature and low relative humidity, indicating high seed moisture favored lipid oxidation as well as vigor deterioration.

Respiratory Activity and Energy Supply

Seed germination, like any growth process, is highly dependent upon respiratory metabolism. This involves the oxidative breakdown of organic storage materials in the seed such as sugars, starches, and lipids. As a result, numerous chemical intermediates are produced which are utilized in biosynthetic reactions essential for germination and seedling growth (Ching, 1972a). These include the synthesis of structural and enzymatic proteins, nucleic acids, and lipids. Respiration also provides available energy in the form of adenosine triphosphate (ATP) which is required by many energy-dependent anabolic reactions.

One approach to evaluating respiratory activity of seeds or seed parts has been the manometric measurement of oxygen consumption or carbon dioxide (CO_2) evolution during imbibition. Employing this technique, Parrish and Leopold (1978) determined oxygen absorption during the early imbibition stage of cotyledons from soybean seeds subjected to various durations of accelerated aging. As aging time increased the rate of oxygen consumption decreased. Average axis weights and root lengths also decreased in a similar manner as oxygen uptake in response to the aging treatments. A more detailed study examining

the effects of accelerated aging on respiratory metabolism of soybean seeds was conducted by Leopold and Musgrave (1980). Their results showed nonaged seeds utilized the cytochrome and alternate pathways in the cotyledons, but only the cytochrome pathway in the axis during the first hour of water uptake. Accelerated aging caused a marked decrease in respiration, particularly through the cytochrome pathway, and there was a shift to the alternate pathway in the axis. Based on these findings they suggested that depressed respiratory activity and the shift in respiration to the less efficient alternate pathway reduced the capacity for energy (ATP) production even more than the lowered respiratory rate would indicate. The consequences of this respiratory change would then be reflected in subsequent germination and growth processes.

The reduced rate of respiratory activity during the initial phase of germination has been shown to persist, in at least naturally aged seeds, through the later stages of germination and seedling development. Wahab and Burris (1971) compared seedling growth characteristics and respiratory activity of soybean seeds aged for 2 years under ambient climatic conditions in Iowa to seeds held for 1 year at 10^{o} C and 4.7% moisture at the same location. Aging resulted in significantly lower rates of oxygen uptake in both the axes and cotyledons throughout 72 hours of germination. This apparent reduction in respiratory activity corresponded to physical indications of vigor deterioration, specifically shorter seedling lengths and slower dry weight accumulation in the axes of aged seeds.

Woodstock and Grabe (1967) demonstrated a similar relationship between seed respiration during early imbibition and subsequent seedling

growth in corn. Respiratory measurements were made during the first 30 hours of imbibition. Seeds previously stored under unfavorable temperature and relative humidity conditions for 4 years consumed less oxygen, but evolved more CO_2 than seeds stored under more favorable conditions. The decrease in respiratory activity in the deteriorated seeds, reflected by oxygen uptake, preceded reduced seedling growth evident 3 to 5 days after planting. No explanation was offered as to why higher quantities of CO_2 were liberated from the aged seeds, although the authors suggested that lower respiratory activity during the early phase of germination may have restricted the amount of ATP available for biosynthetic processes.

Other investigators have examined changes occurring in respiratory substrates during germination with respect to seed aging (Abdul-Baki, 1969a; Anderson, 1970). Work with barley and wheat indicated that increased CO_2 production by accelerated and naturally aged seeds during early germination did not appear to be the result of glucose oxidation. In these experiments, seeds of different vigor levels were incubated in a medium containing ^{14}C-labelled glucose. Aged seeds absorbed the same amount of glucose, but utilized it at a much slower rate and produced less $^{14}CO_2$ than nondeteriorated seeds. However, the total amount of nonradioactive CO_2 produced by the aged seeds greatly exceeded that from seeds which had not undergone aging. A subsequent study revealed poor correlations between respiratory glucose metabolism of whole seeds and indices of vigor deterioration in barley and wheat caused by accelerated aging (Anderson and Abdul-Baki, 1971). This was partially resolved by the finding that after aging embryos differed from endosperm tissue in 3 major aspects. Embryos utilized a much greater percentage of labelled

glucose than the endosperms. In the embryo the percentage of glucose incorporated into ethanol insoluble material declined gradually relative to the loss of vigor, whereas in the endosperm most of the decline preceded any reduction in germination or seedling growth due to the aging treatment. It was also found that the percentage of utilized glucose given off as $^{14}CO_2$ by the embryo either remained unchanged or increased slightly as the seeds deteriorated, but $^{14}CO_2$ produced by the endosperm declined rapidly with increased aging.

In regard to energy availability, respiring seeds produce the majority of ATP within the mitochondria. When mitochondria were isolated from dark-grown seedling axes of new and old soybean seeds, differential phosphorylative efficiency was noted when identical co-factors and substrates were provided (Abu-Shakra and Ching, 1967). The phosphorus to oxygen ratio of mitochondria from the slower growing aged material was 40% to 70% of that from vigorous seedlings of nonaged, indicating reduced ATP production in the former. Uncoupling had most likely occurred in the aged material as oxygen uptake per unit weight of mitochondrial nitrogen was 110% to 140% greater in the older seeds.

The fact that ATP is required as an energy source in order for many biosynthetic reactions to proceed implies that adenylate energy supply plays an important role in facilitating germination and seedling growth. Most types of seeds investigated thus far have been typified by low or undetectable amounts of ATP prior to imbibition (Abernethy et al., 1977; Anderson, 1977; Brown, 1962; Ching and Ching, 1972; Cohn and Obendorf, 1976; Moreland et al., 1974; Obendorf and Marcus, 1974). When identified, the major portion of the adenylate energy pool in dry seeds has generally been shown to be comprised of adenosine

monophosphate (AMP). As the seed beings to imbibe water, the concentration of ATP rises with a drop in AMP content, while adenosine diphosphate (ADP) remains relatively constant (Brown, 1965; Cohn and Obendorf, 1976).

Ching (1973) proposed that an inability to synthesize ATP during the early stages of germination may have been a limiting factor of respiratory metabolism in crimson clover seeds stored for 15 years. Lower quantities of ATP were detected in aged seeds after a 4 hour imbibition period compared to fresh seeds. The ATP content was also positively correlated to differences in germination and seedling growth. Similar results have been previously reported for lettuce seeds subjected to rapid aging conditions (Ching and Danielson, 1972). Using axes from accelerated aged soybean seeds, Anderson (1977) found that the rate of conversion of adenine and adenosine to ATP was reduced as a result of aging. The aging treatment was reflected in decreased incorporation of these compounds into ribonucleic acids (RNA) and a lower rate of protein synthesis within the first 3 hours imbibition. In subsequent work by Styer et al. (1980), the relationship of ATP concentration to germination and seedling vigor of several types of vegetable seeds stored under various conditions was examined. Although germination and seedling vigor decreased as storage time, temperature, and moisture level increased, reduced seed ATP content did not consistently correlate with lower germination or vigor in any species.

Another aspect of adenylate energy metabolism investigated in relation to germination and seedling growth is energy charge. Atkinson (1969) first defined energy charge as half the number of anhydride bound phosphate groups per adenine moiety (energy charge = (ATP) +

0.5(ADP)/(ATP) + (ADP) + (AMP)). According to Atkinson, the energy charge modulates or reflects the activity of various metabolic processes within an organism. Working with animal tissue, he found that when the energy charge was less than 0.5 ATP-regenerating pathways were primarily active, while ATP-utilizing systems predominated with energy charge values greater than 0.5 (Atkinson, 1968). Abernethy et al. (1977), Ching and Ching (1972), and Rodaway et al. (1979) worked with different types of seeds but all found that dry seeds had a low energy charge. Upon imbibition the energy charge increased with increasing ATP and decreasing AMP levels.

Although literature is lacking on the relationship between energy charge and vigor deterioration brought about by seed aging, the concept of energy charge has been studied with other factors affecting germination and seedling growth. Ching and Ching (1972) showed that there are different energy charges in different seed parts and at different stages of seedling development in pine. Energy charges fluctuated between 0.6 at the rapid cell division stage to 0.8 at the fully differentiated stage in the seedling, while the energy charge continually remained around 0.8 in the gametophyte. These results point out that energy metabolism and its possible regulatory effects are compartmentalized in germinating seedlings.

Crompton et al. (1978) assayed phosphorylated adenylates from 3 types of peanut seeds differing in vigor due to their genotypic background. While vigorously growing seedlings had higher levels of ATP than low vigor seedlings, no significant correlation between energy charge and seedling vigor was found. Seedlings from all 3 genotypes maintained high energy charge values throughout germination.

Abernethy et al. (1977) showed a significant difference in the energy charges of unimbibed panicgrass based on seed weight. Heavier seeds had higher energy charge values as well as greater quantities of phosphorylated adenylates than light weight seeds. However, once germination had been initiated, differences in energy charge were no longer evident.

A study of temperature regulation of germinating crimson clover seeds at temperatures of 10^{o}, 20^{o}, and 30^{o} C over a 24 hour period was conducted by Ching (1975). All seeds increased energy charge at approximately the same rate for the first 6 hours. The energy charge in the 30^{o} C lot then declined, but the lots at the other 2 temperatures maintained high energy charge values throughout the remaining 18 hours. The energy charge did not seem to be the sole modulator of biosynthetic processes in these seeds. A greater rate of protein synthesis was observed in seeds imbibed at 30^{o} C which had the relatively lower energy charge. However, total germination was lower with the seeds grown at 30^{o} C.

Changes in Synthetic Rate of Protein and Nucleic Acids

Initiation of new growth and the maintenance of existing cells within imbibing seeds is dependent upon the synthesis of new cellular components. Deterioration due to both accelerated and prolonged aging conditions has been shown to limit the synthesis of various types of cellular materials.

Incorporation of labelled phenylalanine into proteins and uridine into RNA was found to be substantially lower in 15-year-old crimson clover seeds relative to seeds which had not undergone any aging (Ching, 1972b).

Abdul-Baki and Chandra (1977) found that accelerated aging treatments reduced soybean seed germination and growth of excised axes, but did not alter the total nucleic acid content of the axes. The aging treatment did reduce all types of nucleic acid synthesis during the time prior to germination, as well as the incorporation of leucine into protein. Based on their results, they were unable to attribute the decline in protein synthesis in the aged seeds to loss of messenger RNA, but did consider this as a possible cause. Their findings have also been confirmed in a similar study by Anderson (1977).

Van Onckelen et al. (1974) demonstrated with barley seeds that as the time of accelerated aging was increased, the synthetic capacity for various types of RNA in the embryo decreased. Even though aging eventually resulted in loss of viability, RNA synthesis was still observed in the aleurone tissue of seeds that would not germinate, indicating differential loss of synthetic capacity within different parts of the seed.

In corn seeds it was found that the rate of RNA and protein synthesis were higher in embryos subjected to accelerated aging for up to 14 days compared to nonaged embryos (Berjak and Villiers, 1972c). This was interpreted as a compensatory production of these compounds which may have been damaged during the aging process. A decline in protein and RNA synthesis occurred in embryos aged longer than 14 days, suggesting that damage to the synthetic mechanisms had not been repaired during the early stages of imbibition.

Abdul-Baki (1969a) studied protein and polysaccharide synthesis capacities of germinating barley and wheat seeds from 4 successive crop years stored under similar conditions. He noted decreased vigor with

increased age in both types of seeds. A reduced ability to utilize glucose for the synthesis of starch, cellulose, and protein paralleled the loss of vigor. When accelerated aged seeds were compared to the seeds aged over a prolonged storage period, a similar trend in reduced synthetic rates was observed.

Of the various biosynthetic mechanisms which take place in germinating seeds, the components of the protein synthesizing system have perhaps been most closely examined with the purpose of determining the limiting factor responsible for loss of vigor and viability following aging.

Supportive evidence exists that the essential components for protein synthesis are conserved in physiologically mature dry seeds. For example, ribosomes have been shown to be present in dry lettuce seeds (Fountain and Bewley, 1973), caster been seeds (Bewley and Larson, 1979), embryos of wheat (Marcus et al., 1968), pine (Sasaki and Brown, 1971), and rye (Roberts et al., 1973) and peanut cotyledons (Jachymczyk and Cherry, 1968). Preexisting messenger RNA has been isolated from unimbibed wheat (Chen et al., 1968), rice (Bhat and Padayatta, 1974), and pea seeds (Jachymczyk et al., 1974).

It has been established that protein synthesis in viable seeds begins soon after hydration (Anderson, 1977; Rodaway et al., 1979). This newly formed protein is presumably coded for by conserved messenger RNA and translated on ribosomes that have remained stable in dry seed (Spiegel and Marcus, 1975; Weeks and Marcus, 1971), or may also occur through transcription of new messenger RNA (Dobrzanska et al., 1973; Sen and Osborne, 1977).

Although protein synthesis occurs soon after imbibition in viable seeds, impairment of this process appears to occur in aged or nonviable seeds. According to Osborne et al. (1977), failure of the protein synthesizing system may not be the result of energy loss alone as certain components of the protein synthesizing mechanism lose activity while the seed remains in the dry state. Some experimental evidence supports their contention. In dry rye seeds, Roberts et al. (1973) demonstrated breakdown or fragmentation of ribosomal RNA in association with the loss of viability. Similar findings were reported for RNA isolated from ribosomes of unimbibed nonviable pea seed axes (Bray and Chow, 1976). In both cases, ribosomes from these nonviable axes had an impaired ability to support in vitro assembly of amino acids into polypeptides. In addition, the activity of enzymes responsible for binding the transfer RNA were reduced relative to that observed in viable seeds. It was further demonstrated that nonviability in rye embryos was associated with decreased precursor incorporation into all classes of RNA (Sen and Osborne, 1977). The authors hypothesized that reduced RNA synthesis may be partially due to an impaired deoxyribonucleic (DNA) template. A faulty DNA template may then result in nonfunctional or "nonsense" RNA.

Subsequent work by Cheah and Osborne (1978) supports this theory. They found that fragmentation of DNA had occurred with the loss of rye seed viability. Greater quantities of nucleases were isolated from the nonviable embryos compared to viable embryos. On the basis of these results, they suggested that activity of these enzymes may be slow and gradual in dry seeds but may have caused the breaks in DNA. The authors also stated that repair of DNA damage seems unlikely during the aging of

dry seeds and an accumulation of breaks in DNA could eventually reach a level too high to be repaired at germination. In agreement with this idea Villiers (1974) found that when lettuce seeds were stored fully imbibed, but kept dormant, fewer chromosome aberrations occurred compared to dry-stored seeds. The hydrated seeds also showed a higher germination capacity and a lower degree of seedling morphological abnormalities, indicating that DNA repair mechanisms were functional in these seeds during storage.

CHAPTER II
VIGOR IN SOYBEAN SEEDS AFTER OPTIMUM STORAGE AND ACCELERATED AGING

Research emphasis has been directed towards developing accelerated aging as a feasible method for evaluating seed storability and vigor of soybeans and other crops (Delouche and Baskin, 1973; Egli et al., 1979; McDonald, 1977; McDonald, 1980; McDonald and Phaneendranath, 1978; Tao, 1979). The technique has also been used as a convenient way to rapidly induce seed deterioration for the study of metabolic factors associated with vigor loss (Abdul-Baki and Chandra, 1977; Anderson, 1977; Parrish and Leopold, 1978; Priestley and Leopold, 1979; Stewart and Bewley, 1980).

Seed vigor can deteriorate during storage (Barton, 1961; Ching et al., 1959; Owen, 1957). Factors affecting the loss of vigor during storage include not only seed moisture and the temperature of the storage environment, but also the initial vigor of the seed (Delouche and Baskin, 1973; Egli et al., 1979). Seeds of low initial vigor have been shown to deteriorate more readily than seeds with high initial vigor (Barton, 1941).

When studies are undertaken employing accelerated aging as a method for inducing vigor deterioration, consideration should be given to the amount of vigor loss taking place in the seed lot in storage during the course of the investigation. Therefore this study was undertaken to determine the effect of accelerated aging as a vigor determinant of soybean seeds after different periods of "optimum" storage.

Materials and Methods

Soybean seeds (Glycine max (L.) Merr., cv. 'Bragg') were obtained from the breeding line of Dr. K. Hinson, Agronomy Department, University of Florida, Gainesville, Florida. The seeds were planted in a single block in a Kanapaha fine sandy soil at the Horticultural Unit, Gainesville, Florida, on June 22, 1978. Cultural practices were based upon Florida recommendations (Hinson, 1969). Plants were harvested on November 22, 1978, when the average seed moisture content was 14.5%. This was determined from 4 groups of 100 seeds randomly selected from plants in the field. Each group was weighed, dried at 100° C for 48 hours, and reweighed. Seed moisture content was expressed as a percentage of the initial weight of the seeds from the field. Following mechanical threshing, the seeds were dried for 48 hours at 32° C to 7.5% moisture. The seeds were then stored in sealed plastic containers at approximately 10° C and 45% relative humidity.

A portion of seeds was removed from storage annually for 3 years after the first year of storage. Each year, seeds were uniformly sized by screening to a diameter of 7 mm and divided into 2 lots. One lot was subjected to accelerated aging according to the method of McDonald and Phaneendranath (1978). Samples of 200 nondamaged seeds were weighed and placed in plastic boxes (11.0 x 11.0 x 3.5 cm), containing 40 ml of deionized water. The seeds were supported in a single layer 2.5 cm over the water on a wire-mesh tray (Figure II-1). The boxes were covered with lids, sealed with tape, and held at 41° C for 64 hours. Immediately after removal from the boxes, each sample was reweighed to determine the increase in seed moisture content. Only samples that increased seed moisture to 22.5% to 25.5% were retained for experimental

Figure II-1. Plastic box with wire tray used for subjecting soybean seeds to the accelerated aging treatment.

use. The seed samples were then redried to their initial weight on wire-mesh screens at 27° C in a low humidity chamber.

Vigor evaluations were conducted yearly with accelerated aged seeds and those which did not receive the accelerated aging treatment, hereafter referred to as aged and nonaged seeds, respectively. Four replicates of 50 seeds were each placed between 2 layers of moist paper towels, rolled up with a sheet of waxed paper to reduce evaporation, and placed upright in a 25° C incubator. Germination counts were made 24, 48, 72, and 96 hours after the start of imbibition. A seed was considered germinated when the radicle had proturded from the seed coat to a length of 3 mm. Additional deionized water was applied to the towels at each 24 hour interval to maintain adequate moisture.

Vigor was assessed on the basis of germination percent, germination rate, and several parameters of seedling growth and development. The rate of germination was expressed as the average number of days for a seed to germinate (ADG) and was determined by the emergence rate formula of Smith and Millet (1973). Calculations were made using the following equation:

$$ADG = \frac{\sum_{n=1}^{n=c} X_n (n)}{x}$$

Where X_n = the number of seeds germinating on day n.

n = the day on which the germination count was made.

c = the duration of the test in days.

x = the total number of seeds germinating over the entire germination period.

Thus, the lower the ADG, the faster the rate of germination.

After the final germination count was recorded the percentage of high vigor seedlings was determined. High vigor seedlings were those

having a hypocotyl and radicle 5 cm or longer which were morphologically normal at this length (Woodstock, 1976). The following criteria, as specified by the Association of Official Seed Analysts (Anonymous, 1970), were used to determine a morphologically normal seedling:

1. A vigorous primary root or set of secondary roots sufficient to anchor the seedling when grown in soil or sand.
2. A sturdy hypocotyl with no open breaks or lesions extending into the central conducting tissue.
3. At least 1 attached cotyledon.
4. An epicotyl with at least 1 primary leaf and an intact terminal bud.

Axis lengths and fresh weights of the cotyledons and axes of all seedlings within a replication were measured at the end of the 96 hour germination period. Axis and cotyledon dry weights were recorded after oven-drying at 70^{o} C for 48 hours.

Results and Discussion

Maintaining soybean seeds at approximately 10^{o} C and 45% relative humidity for as long as 3 years had no effect on germination and only a minimal effect on vigor deterioration (Table II-1). No evidence of storage deterioration was apparent from germination rates as ADG values of nonaged seeds did not change. Other investigators have found that initial germinability of soybean seeds held under similar temperature and moisture conditions did not decline during 10 years of storage (Toole and Toole, 1946).

A reduction in axis fresh weight from nonaged seeds was observed by the third year of storage. However, this change in fresh weight was not reflected by reduced axis dry weight or axis length which were

Table II-1. Effect of storage time and accelerated aging on several parameters of germination performance and seedling growth associated with soybean seed vigor.

Seed Treatment	Storage Time (years) 1	2	3
		Germination (percent)	
Nonaged	100a[z]	100a	100a
Aged	98a	97a	97a
	ns[y]	*	*
		Germination Rate (ADG)	
Nonaged	1.9a	1.9a	1.9a
Aged	1.9a	1.9a	2.2b
	ns	ns	*
		Axis Fresh Weight (mg/axis)	
Nonaged	346.3a	341.1a	312.4b
Aged	142.8a	142.2a	113.2b
	*	*	*
		Axis Dry Weight (mg/axis)	
Nonaged	24.6a	23.8a	24.7a
Aged	15.7a	13.2b	10.6c
	*	*	*
		Axis Length (mm/axis)	
Nonaged	138.1b	144.5ab	146.6a
Aged	64.2a	58.1ab	48.7b
	*	*	*
		Cotyledon Fresh Weight (mg/cotyledon pair)	
Nonaged	344.0a	308.0b	347.6a
Aged	322.1b	328.3ab	338.6a
	ns	*	*
		Cotyledon Dry Weight (mg/cotyledon pair)	
Nonaged	114.8ab	111.3b	117.5a
Aged	119.6b	127.8a	129.9a
	ns	*	*

Table II-1--Continued

Seed Treatment	Storage Time (years)		
	1	2	3
	High Vigor Seedling (percent of germinated seeds)		
Nonaged	96a	91b	84b
Aged	54a	53a	48a
	*	*	*

[z]Mean separation in rows by Duncan's multiple range test, 5% level.

[y]Mean separation in columns by Duncan's multiple range test: ns = not significant; * = significant at the 5% level.

essentially the same for 1, 2, and 3-year-old nonaged seeds, unless the seeds were aged.

Differences in cotyledon fresh and dry weights from 4-day-old seedlings of nonaged seeds failed to identify any definite trends in vigor deterioration that could be attributed to the storage duration. Although seeds used in all 3 vigor evaluation tests were similar in size, differences in seed density may have resulted in cotyledon fresh and dry weight variability.

Of the various indices of nonaged seedling growth, differences in the percentage of high vigor seedlings, as outlined by Woodstock (1976), were the only clear indication that vigor deterioration may have occurred during storage. The number of seedlings classified as high vigor significantly declined with each successive year the nonaged seeds were held in storage. This did not occur, however, after the seeds were aged.

Not only did accelerated aging reduce several parameters of seedling vigor relative to nonaged seeds, but it also provided a more pronounced indication that some deterioration of seed vigor had occurred over the 3 years of storage. Accelerated aging for 64 hours did not cause a substantial reduction in germination. This is in agreement with the results of McDonald (1977) and others (Woodstock and Tao, 1981), who found that soybean seed germination did not decline unless the seeds were aged for longer periods of time. However, such findings are highly dependent on the cultivar and the initial vigor of the seed lot prior to accelerated aging (Delouche and Baskin, 1973; Egli et al., 1979). Although the germination rate of aged and nonaged seeds was the same for the first 2 years of storage, a significantly higher ADG value,

denoting a slower rate of germination, was observed for 3-year-old seeds following accelerated aging. This suggested that vigor deterioration may have commenced. Data on aged axis growth from successive vigor evaluations also support this assumption. Previous work has established that accelerated aging generally results in reduced axis or seedling growth (Burris et al., 1969; Edje and Burris, 1970; McDonald, 1977; Roos and Manalo, 1971; Woodstock and Grabe, 1967; Woodstock and Tao, 1981). In addition to this observation, it was found that the decrease in aged axis length and axis dry weight closely corresponded to increased storage time. However, there was no statistically significant reduction in the percent of high vigor seedlings with time.

Cotyledon fresh weights of aged seeds were variable and followed no trend in regard to a change in vigor during the storage period. Significantly higher cotyledon dry weights were observed after 2 and 3 years of storage relative to 1-year-old seeds subjected to accelerated aging and those which did not receive the stress treatment. The higher cotyledon dry weights from seedlings of aged seeds from the last 2 vigor evaluations may have reflected a decline in the utilization of energy reserve materials during the 4 day germination period.

Summary

Successive vigor evaluation tests conducted with nonaged seeds showed only slight evidence that deterioration of soybean seeds took place under favorable storage conditions. This was evidenced by a decline in the percentage of high vigor seedlings with each year the seeds were held in storage. Similar tests were made with seeds from the same lot after accelerated aging stress had been imposed. Results

of these evaluations with aged seeds indicated that slight vigor deterioration had been initiated during storage; however the percentage high vigor seedlings remained unchanged after 3 years of storage. A progressive decline in axis growth (dry weight and length) was observed as storage time increased. Even though slight deterioration might have occurred under favorable storage conditions, the extent of vigor loss was minute compared to that induced by the accelerated aging treatment.

CHAPTER III
SOLUTE LEAKAGE FROM SEEDS AND AXES OF SOYBEAN AS INFLUENCED BY ACCELERATED AGING

Previous studies have indicated that seed vigor deterioration may be related to changes in cell membranes (Abdul-Baki, 1980; Harman and Granett, 1972; Koostra and Harrington, 1969; Matthews and Bradnock, 1968; Parrish and Leopold, 1978). In dry seeds, cell membranes are thought to be disorganized (Abdul-Baki, 1980; Simon, 1974; Simon and Raja Harun, 1972). Upon imbibition there is a brief period before membrane continuity is established during which time solutes leak out of the cells (Parrish and Leopold, 1977). Low vigor seeds have been shown to leak greater quantities of electrolytes and other cellular constituents during this time (Ching and Schoolcraft, 1968; Knypl, 1979). In addition to adverse effects on metabolic processes, continued leakage can predispose the seed to pathogenic attack (Harman and Granett, 1972; Keeling, 1974).

Parrish and Leopold (1978) found that the loss of soybean seed vigor due to accelerated aging was associated with increased electrolyte leakage from excised cotyledons during the first few minutes of imbibition. They hypothesized that vigor deterioration of the accelerated aged seeds was the result of the failure of membranes to reorganize upon hydration. The present study was undertaken to determine how accelerated aging influences electrolyte leakage from intact soybean seeds and excised axes. The effect of the stress treatment on the

retention of stored reserve materials was also investigated as these compounds play an essential role in early seedling growth.

Materials and Methods

Time-course leachate tests were conducted to assess membrane permeability and the loss of stored reserve materials upon imbibition from accelerated aged soybean seeds and axes. Evaluations were made relative to seeds which were not subjected to the aging treatment. The seed lot and methodology for accelerated aging were the same as those identified in Chapter II.

For the leachate test with whole seeds, replicated samples of 25 seeds were weighed and placed in 125 ml Erlenmeyer flasks. A 100 ml volume of deionized water was added to each flask. Flasks containing only water were prepared as internal controls. The flasks were stoppered and placed in a 25° C incubator for 0.25, 0.50, 1, 3, 6, 12, and 24 hours. At the end of each leaching period samples were gently swirled. Seeds and particulate matter in the leachate solutions were removed by decanting the contents of each flask through a layer of 5.5 cm Whatman #1 filter paper in individual glass funnels. The leachate solutions were then frozen until further use. Immediately after collecting the leachate solutions, seed samples were blotted with tissue paper to remove surface moisture and weighed. The seeds were dried at 70° C for 48 hours, reweighed, and moisture contents determined.

Measurements of electrolyte conductivity and potassium ion (K^+) concentrations were determined on aliquots of the leachate solutions to evaluate membrane permeability. Electrolyte conductivity was measured with a Lab-Line Lectro Mho-Meter and K^+ concentrations by flame spectrophotometry (Chapman and Pratt, 1961; Woodstock, 1976). Data

values were expressed as μmhos per seed and parts per million (ppm) per seed, respectively.

Additional aliquots of the leachate solutions were assayed for concentrations of the following stored reserve materials lost from the seeds: carbohydrates, amino acids, and proteins. Carbohydrates were determined by the anthrone technique using glucose as a standard (Umbreit et al., 1959). The ninhydrin technique of Moore (1968) was used to measure amino acid concentrations. Standards were prepared from L-leucine. Protein concentrations were determined by the method of Lowry et al. (1951) as modified by Cooper (1977) with bovine serum albumin used as the standard. The carbohydrate, amino acid, and protein concentrations were calculated as ppm per seed. In addition, all leachate data values were expressed as percentages of their respective Total Potential Leakage (TPL). The TPL values for various electrolytes and the stored reserve materials were determined on aged and nonaged seeds by holding samples of 25 seeds in 100 ml of deionized water at 80^{o} C for 30 minutes. This treatment disrupted cell membranes as well as killed the seeds. The solutions containing the heat-stressed seeds were cooled and held at 25^{o} C for 24 hours. The leachate solutions were then filtered and analyzed for electrolyte conductivity and concentrations of K^{+}, carbohydrates, and amino acids as previously described.

For the leachate test with embryonic axes, samples of 20 axes excised from aged and nonaged seeds were used for leaching periods up to 1 hour. Ten axes per sample were used thereafter. All samples were placed in individual 20 ml glass vials containing 5 ml of deionized water. Similar procedures as described for the seed leachate test were then followed. The axis TPL values of electrolyte conductivity, K^{+},

carbohydrate, and amino acid concentrations were determined on a sample of size of 10 axes. Axis leachate data were expressed on a per axis basis and as percentages of the appropriate TPL value.

Results and Discussion

Both aged and nonaged seeds leaked electrolytes immediately upon immersion in water (Table III-1). This response has been found to be typical of many types of seeds during the early phase of imbibition (Abdel Samad and Pearce, 1978; Bramlage et al., 1979; Larson, 1968; Parrish and Leopold, 1977; Perry and Harrison, 1970; Schroth et al., 1966; Simon and Raja Harun, 1972). The early period of imbibition is thought to be the time when seed membranes become rearranged from a porous state to intact, selectively permeable barriers (Bewley and Black, 1978; Simon, 1974). Differences in seed membrane permeability that could be attributed to the accelerated aging treatment were apparent after the first 30 minutes of imbibition (Table III-1). Prior to this time, the seed coat may have partially retarded the diffusion of electrolytes (Abdel Samad and Pearce, 1978; Duke and Kakefunda, 1981). Using excised soybean cotyledons, thus eliminating the influence of the seed coat, Parrish and Leopold (1978) found that accelerated aging resulted in a greater rate of electrolyte loss after only 10 minutes of imbibition.

By the third hour of leaching, the margin between conductivity readings of aged and nonaged seeds greatly increased (Table III-1). Thereafter, conductivity differences due to the stress treatment became progressively larger with time, suggesting that impairment of membrane function had occurred as a result of accelerated aging. This assumption is further evidenced by higher K^+ leakage from the aged seeds after 3

Table III-1. Effect of accelerated aging on the loss of electrolytes and stored reserve materials from soybean seeds during leaching.

Seed Treatment	Leaching Time (hours) .25	.50	1	3	6	12	24
			Electrolyte Conductivity (μmhos/seed)				
Nonaged	1.44	2.24	3.20	4.20	5.04	6.60	10.04
Aged	1.60	2.36	3.40	5.16	7.76	10.68	16.64
	ns[z]	*	*	*	*	*	*
			Potassium (ppm/seed)				
Nonaged	0.44	0.72	0.96	1.36	1.56	1.92	3.04
Aged	0.48	0.68	1.00	1.64	2.32	3.24	5.40
	ns	ns	ns	*	*	*	*
			Carbohydrates (ppm/seed)				
Nonaged	0.16	0.24	0.36	0.72	0.80	1.32	1.40
Aged	0.24	0.24	0.52	1.28	2.24	2.56	2.80
	ns	ns	ns	*	ns	*	*
			Amino Acids (ppm/seed)				
Nonaged	0.08	0.08	0.08	0.08	0.12	0.16	0.20
Aged	0.12	0.12	0.20	0.32	0.40	0.52	0.76
	ns	ns	*	*	*	*	*

[z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant 5% level.

hours of imbibition (Table III-1). From then on, corresponding patterns for K^+ leakage and electrolyte conductivity were observed.

Several investigators have demonstrated that a faster rate of imbibition can lead to cellular rupture resulting in greater electrolyte leakage (Larson, 1968; Powell and Matthews, 1978). In the present study, greater electrolyte leakage did not appear to be the result of a more rapid rate of water uptake by the aged seeds. Moisture contents of the aged seeds did not significantly differ from nonaged seeds throughout the course of the test (Table III-2).

After 3 hours of leaching more carbohydrates were lost from the aged seeds than nonaged seeds (Table III-1). In contrast, Ching and Schoolcraft (1968) were unable to discern a definite trend in carbohydrate leaching of rye seeds which differed in germinability and vigor.

A greater quantity of amino acids, measured in ppm per seed, was lost from aged seeds after 1 hour of leaching (Table III-1). However, when the data were expressed as percentages of the TPL of amino acids, differences in amino acid leakage between aged and nonaged seeds were not evident until the third hour of leaching (Table III-3). These findings suggested that the amount of leached amino acids might have been biased by differences in endogenous amino acid levels in the seeds prior to imbibition. Other investigators have shown an increase in free amino acids in rye and sunflower seeds during prolonged storage under adverse temperature and humidity conditions (Ching and Schoolcraft, 1968; Halder and Gupta, 1980). Although protein hydrolysis may have taken place during the 30 minute heat treatment used to determine the amino acid TPL values, results showed that more amino acids were lost from the aged seeds (Table III-4). This observation indicated that protein might have been degraded during the accelerated aging treatment.

Table III-2. Changes in seed and excised axis moisture content during leaching.

Seed Treatment	Leaching Time (hours)						
	.25	.50	1	3	6	12	24
	Seed Moisture Content (mgH_2O/mg dry weight)						
Nonaged	0.3	0.4	0.6	0.8	1.2	1.4	1.6
Aged	0.3	0.4	0.6	0.9	1.2	1.4	1.6
	Axis Moisture Content (mg H_2O/mg dry weight)						
Nonaged	1.0	1.4	1.6	1.8	1.9	2.2	2.8
Aged	1.0	1.4	1.7	1.9	2.1	2.2	2.8

Table III-3. Effect of accelerated aging on the relative loss of electrolytes and stored reserve materials from soybean seeds during leaching. Means are expressed as percentages of their respective Total Potential Leakage (TPL) values presented in Table III-4.

Seed Treatment	Leaching Time (hours)						
	.25	.50	1	3	6	12	24
			Electrolyte Conductivity (percent of TPL)				
Nonaged	2	3	4	6	7	9	14
Aged	2	3	5	7	11	15	23
	ns[z]	ns	*	*	*	*	*
			Potassium (percent of TPL)				
Nonaged	2	3	6	6	7	9	14
Aged	2	3	5	8	11	15	25
	ns	ns	ns	*	*	*	*
			Carbohydrates (percent of TPL)				
Nonaged	1	2	3	6	6	10	11
Aged	2	2	4	10	17	20	22
	ns	ns	ns	*	*	ns	*
			Amino Acids (percent of TPL)				
Nonaged	1	1	2	2	2	3	4
Aged	1	1	2	4	5	6	8
	ns	ns	ns	ns	*	*	*

[z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant at the 5% level.

Table III-4. Total Potential Leakage values of various seed leachate components after heating seeds at 80° C for 30 minutes in 100 ml of deionized water.

Leachate Component	Seed Treatment	
	Nonaged	Aged
Electrolyte Conductivity (μmhos/seed)	72.7a[z]	72.7a
Potassium (ppm/seed)	21.1a	21.5a
Carbohydrates (ppm/seed)	12.9a	12.8a
Amino Acids (ppm/seed)	5.9a	8.8b

[z]Mean separation in rows by Duncan's multiple range test: 5% level.

The effect of accelerated aging on the leaching of storage reserve materials appeared to be primarily limited to lower-molecular-weight compounds, as proteins in the leachate solutions of aged and nonaged seeds were undetectable throughout the 24 hour test period (data not presented).

Differences in electrolyte conductivity of axes excised from aged and nonaged seeds became apparent much sooner than the differences observed with intact seeds (Table III-5). There was a greater rate of hydration in the axes relative to the intact seeds which may have allowed for the more rapid release of solutes from the axes (Table III-2). The faster imbibition rate of the axes may have been due, in part, to the absence of a protective barrier such as the coat. Differences in the composition and amount of hydrating substances in the seed tissue could also have affected the rate of water uptake (Mayer and Poljakoff-Mayber, 1975). Blacklow (1972) found that when the water content of dent corn seeds reached 75%, the water content of the embryo was 261%, whereas the remainder of the seed had a water content of 50%.

After 15 minutes of imbibition, leachate solutions of aged axes had higher electrolyte conductivity than those of nonaged axes indicating axis membrane permeability was increased as a result of the accelerated aging treatment (Table III-5). A similar relationship for K^+ leakage was observed between aged and nonaged seed axes immediately after imbibition had begun (Table III-5). Axes from nonaged seed lost electrolytes K^+ during the entire leaching period, but the losses were always less than those from the aged axes.

There were no differences in the moisture contents between aged and nonaged axes throughout the test (Table III-2). Therefore, the

Table III-5. Effect of accelerated aging on the loss of electrolytes and stored reserve materials from soybean seed axes during leaching.

Seed Treatment	Leaching Time (hours) .25	.25	1	3	6	12	24
			Electrolyte Conductivity (μmhos/axis)				
Nonaged	3.2	4.1	5.3	10.4	14.0	18.6	25.8
Aged	4.8 *[z]	5.5 *	7.2 *	12.7 *	15.4 *	22.2 *	37.2 *
			Potassium (ppm/axis)				
Nonaged	.8	1.0	1.5	2.4	3.9	5.6	6.5
Aged	1.2 *	1.5 *	1.9 *	3.6 *	6.1 *	6.5 *	8.9 *
			Carbohydrates (ppm/axis)				
Nonaged	8.1	11.0	14.9	18.6	25.9	24.5	29.4
Aged	7.9 ns	10.0 ns	13.1 ns	22.0 ns	24.1 ns	33.5 *	40.2 *
			Amino Acids (ppm/axis)				
Nonaged	1.1	1.6	1.8	2.3	3.9	4.4	8.6
Aged	1.8 *	2.2 *	3.0 *	4.2 *	5.5 *	8.5 *	16.0 *

[z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant at 5% level.

rate of imbibition was not a factor influencing the differential loss of electrolytes from aged and nonaged axes.

The increased membrane permeability of aged axes was not as readily reflected by carbohydrate leaching as it was by electrolyte loss (Table III-5). The carbohydrate concentration in the leachate solutions of aged axes did not exceed that of nonaged axes until 12 hours after the start of imbibition, well past the time considered necessary for membrane reorganization (Simon, 1974). The TPL values of carbohydrates from aged and nonaged axes did not differ significantly, thus discounting a compositional effect on axis carbohydrate leakage (Table III-6).

Comparable to the results with intact seeds, higher concentrations of amino acids were found in the leachate solutions of axes from aged seeds (Table III-5). Endogenous amino acid levels again appeared to be higher in the aged material before imbibition, based on the higher amino acid TPL value found for aged axes (Table III-6). Despite this possibility, leached amino acids still remained greater in aged axes than nonaged axes when amino acids in the leachate solutions were calculated as percentages of the TPL of amino acids (Table III-7).

Measurable amounts of protein were not leached from the axes of either aged or nonaged seeds (data not presented). Nevertheless, the greater loss of carbohydrates and amino acids from the axes of accelerated aged seeds may be an important factor contributing to soybean seed vigor deterioration. Leaching of reserve materials from the axes of aged seeds was much more pronounced than the loss from intact aged seeds (Tables III-3, 7). This greater loss of amino acids and carbohydrates from the axes probably occurs in the intact seed. However, these losses may be masked by the seed coat barrier and the relatively

Table III-6. Total Potential Leakage values of various axis leachate components after heating axes at 80° C for 30 minutes in 5 ml of deionized water.

Leachate Component	Seed Treatment	
	Nonaged	Aged
Electrolyte Conductivity (μmhos/axis)	42.0a[z]	43.0a
Potassium (ppm/axis)	12.6a	12.9a
Carbohydrates (ppm/axis)	111.9a	101.1a
Amino Acids (ppm/axis)	25.8a	29.6b

[z]Mean separation in rows by Duncan's multiple range test: 5% level.

Table III-7. Effect of accelerated aging on the relative loss of electrolytes and stored reserve materials from soybean seed axes during leaching. Means are expressed as percentages of their respective Total Potential Leakage (TPL) values presented in Table III-6.

Seed Treatment	Leaching Time (hours)						
	.25	.50	1	3	6	12	24
			Electrolyte Conductivity (percent of TPL)				
Nonaged	8	10	13	25	33	41	61
Aged	11	13	17	30	39	52	86
	*[Z]	*	*	*	*	*	*
			Potassium (percent of TPL)				
Nonaged	6	8	12	19	31	44	53
Aged	9	12	15	28	47	51	69
	*	*	*	*	*	*	*
			Carbohydrates (percent of TPL)				
Nonaged	7	10	13	17	23	22	26
Aged	8	10	13	22	24	33	38
	ns	ns	ns	ns	ns	*	*
			Amino Acids (percent of TPL)				
Nonaged	4	6	7	9	15	17	33
Aged	6	7	10	14	19	29	54
	*	*	*	*	*	*	*

[Z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant at the 5% level.

small size of the axis in comparison to the whole seed. Decreased concentrations of axis reserve materials due to leaching may adversely affect the activity of catabolic and anabolic processes during the early stages of imbibition by reducing substrate availability. Consequently, a general decline in metabolic activity may retard seedling growth. Accelerated aging also promoted the leaching of amino acids and carbohydrates from the intact seed which is primarily composed of cotyledon tissue. Therefore, the total amount of reserve materials available for seedling development was further depleted as a result of the accelerated aging treatment.

Summary

Leachate tests comparing accelerated aged seeds and axes to nonaged seeds and axes indicated that the stress treatment adversely affected solute leakage upon imbibition. Measurements of electrolyte loss during leaching suggested that membrane permeability increased following accelerated aging. As a consequence, there was a tendency for greater quantities of stored reserve materials to be lost from aged seeds and axes during hydration. The percent of solute loss was much higher in axes than in intact seeds. Amino acids readily leached from aged material but appeared to be influenced by higher endogenous levels. Increased amounts of carbohydrates were also lost, but differences due to the accelerated aging treatment were not observed as readily as the loss of electrolytes. The rate of imbibition did not appear to influence the loss of electrolytes or any of the storage reserve materials detected in the leachate solutions relative to the aging treatment.

CHAPTER IV
CHANGES IN METABOLIC PROCESSES IN RELATION TO VIGOR DETERIORATION OF SOYBEAN SEEDS AFTER ACCELERATED AGING

The obvious physical signs of seed vigor deterioration are often evidenced by delayed germination and a slower rate of seedling growth. In contrast, the physiological basis of seed vigor loss and the relationship of impaired metabolic function to reduced seedling growth are much less apparent. For vigorous germination and seedling development to occur, it is essential that many interrelated metabolic systems become operational soon after the seed has begun to imbibe water.

There is supportive evidence linking vigor deterioration to the impairment of various catabolic as well as anabolic processes in seeds during the first few hours of imbibition. Parrish and Leopold (1978) and others (Leopold and Musgrave, 1980) reported that imbibed but ungerminated soybean seeds previously subjected to accelerated aging exhibited suppressed respiratory activity. Similar results have been reported for naturally aged corn seeds (Woodstock and Grabe, 1967). In the same sense, vigor deterioration in barley and wheat seeds has been positively correlated with an inability of the embryo and endosperm to effectively utilize respiratory substrates. Deterioration of vigor in lettuce seeds (Ching and Danielson, 1972), crimson clover seeds (Ching, 1973), and excised soybean axes (Anderson, 1977) has been associated with reduced levels of ATP. Depressed energy charge levels have also been found to be directly related to reduced vigor in imbibed soybean axes (Anderson, 1977) and wheat seeds (Ching and Kronstad, 1972).

Decreased rates of protein and nucleic acid synthesis have been found in imbibed axes of accelerated aged soybean seeds (Anderson, 1977; Abdul-Baki and Chandra, 1977). These findings have also been extended to deteriorated seeds of crimson clover (Ching, 1973), rye (Roberts and Osborne, 1973), barley, wheat (Abdul-Baki, 1969a), and the axes of pea seeds (Bray and Chow, 1976). Abdul-Baki (1969a) reported that polysaccharide synthesis in imbibed wheat and barley seeds declined concomitantly with vigor deterioration.

While the previously mentioned studies have reported several metabolic disorders in deteriorated seeds during the first few hours of imbibition, no attention has been directed towards determining the extent to which these processes remain impaired during seedling early growth. Such information would provide a more thorough understanding of the physiological basis of seed vigor deterioration and therefore was the overall objective of the present study. To accomplish this objective, a series of 5 time-course experiments were conducted comparing the growth and development of accelerated aged and nonaged soybean seeds in relation to the following: (1) axis and cotyledon respiratory activity, (2) axis energy charge levels and axis phosphorylated adenylate contents, (3) changes in metabolic reserve materials in the axis and cotyledons, (4) axis protein synthesis, and (5) axis root tip cell division activity. Differences due to aging treatment might then be correlated to causal factors of vigor deterioration.

Materials and Methods

All experiments were conducted with soybean seeds which originated from the seed lot identified in Chapter II. The method for accelerated aging was the same for all experiments and is described in Chapter II.

If necessary, accelerated aged seeds were stored at 10° C and 45% relative humidity but for no longer than 10 days before use in any experiment. Nonaged seeds were maintained under similar storage conditions.

Experiment 1. Respiratory Activity Changes

The respiratory rates of aged and nonaged seeds were determined by measuring oxygen uptake on a Gilson single valve differential respirometer following standard manometric techniques (Umbreit et al., 1959). Separate measurements were made for axes and cotyledons.

Replicates of 5 aged and nonaged seeds were imbibed or germinated at 25° C for specific times using similar methodology as described in Chapter II. At 0, 24, 48, 72, and 96 hours, seeds or seedlings were dissected into axes and cotyledons which were placed into individual 15 ml Warburg respirometer flasks. Three milliliters of distilled water had been previously added to the main chamber of the flasks containing the cotyledons. The same volume of a 1.0% (weight/volume) sucrose solution was included in the flasks containing the axes. A filter paper wick and 0.4 ml of a 10% (weight/volume) KOH solution was added to the center wells of all flasks to absorb CO_2. The flasks were secured to the respirometer and continuously agitated at 100 cycles per minute in a 25° C water bath. After a 20 minute period for temperature equilibration, manometer valves were closed. The amount of oxygen consumed per flask was read directly in μl after 15 minutes. Initial respiratory rates (Time 0) were made by measuring oxygen uptake during the latter half of the first hour of imbibition. Two flasks, prepared similarly as those for the axes and cotyledons but without seed material, were included with each determination. These flasks served as thermobarometers to correct for pressure changes due to temperature fluctuations.

Cotyledon and axis fresh weights and axis lengths were recorded after removal of the seed parts from the flasks and blotting with tissue paper. Axes and cotyledons were dried at 70° C for 48 hours before dry weights were recorded.

Experiment 2. Changes in Axis Energy Charge and Axis Phosphorylated Adenylates

Samples of 10 aged or nonaged seeds were imbibed or germinated at 25° C as previously described in Chapter II. At 24 hour intervals for 96 hours, samples were taken for measurement of axis phosphorylated adenylate (ATP, ADP, and AMP) concentrations from which energy charge values were determined.

Prior to extraction of the phosphorylated adenylates, seedling fresh weights and average axis lengths were measured after seed coat removal. Axes were dissected from the cotyledons and rapidly frozen in test tubes immersed in liquid nitrogen. The cotyledons were then weighed. The axis fresh weight was calculated by the difference in total seedling fresh weight minus the cotyledon fresh weight.

The frozen axes were shattered into small pieces with a plastic rod before extracting and determining the concentrations of phosphorylated adenylates based on the procedure of Cohn and Obendorf (1976).

Phosphorylated adenylates were obtained from axes of unimbibed seeds by grinding 10 excised axes into a fine powder using a mortar and pestle. The extraction procedure was the same as that employed with the hydrated seeds.

Phosphorylated adenylates were extracted in 10 ml boiling distilled water added to each test tube. The extract solutions were kept at 100° C in a boiling water bath for 10 minutes. Glass marbles were placed on

the tops of the test tubes to minimize evaporation. After approximately 5 minutes of boiling, the axis tissues were ground by hand in the water bath using a plastic grinding bit to aid in extraction.

Immediately after boiling, sample extracts were cooled and kept on ice. A 2 ml volume of buffer containing 0.5 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 0.5 M magnesium acetate (pH 7.5) was added to each sample to aid in sedimentation of fatty material. After centrifugation at 20,000 x g for 10 minutes at 4^{o} C, the extract solutions were decanted and diluted to 20.0 ml with distilled water and held on ice.

An 0.8 ml quantity of the extract solutions was added to each of the following reaction mixtures which were prepared fresh daily:

A. For ATP determinations: 0.1 ml of buffer (pH 7.5) containing 0.1 M HEPES and 0.1 M magnesium acetate, and 0.1 ml distilled water.

B. For ATP + ADP determinations: 0.1 ml of buffer similar to mixture A and 0.1 ml of a solution containing 20 μg of pyruvate kinase and 500 nmoles of trisodium phosphoenolpyruvate necessary to convert ADP in the extract to ATP.

C. For total adenosine phosphates (ATP + ADP + AMP) determinations: 0.1 ml of buffer similar to mixture A and 0.1 ml of a solution containing 20 μg of pyruvate kinase, 500 nmoles of trisodium phosphoenolpyruvate, and 20 μg of adenylate kinase necessary to convert ADP and AMP in the extract to ATP.

The extract solutions were then vortexed and incubated for 15 minutes in a 30^{o} C water bath. To inactivate the enzymes in mixtures B and C, all extract solutions were held in a 100^{o} C water bath for 15 seconds, then cooled, and kept on ice until assaying.

Concentrations of ATP in the extract solutions were determined photometrically by the luciferin-luciferase assay described by St. John (1970). Dry firefly extract containing the luciferin-luciferase enzyme

complex was reconstituted by adding 5.0 ml of cold distilled water per prepared vial. Included in the firefly extract solution were 0.05 M potassium arsenate and 0.02 M magnesium sulfate. To deplete endogenous ATP in the firefly extract, the solution was held at 4^{o} C for 16 hours before assaying. Dual 0.4 ml volumes of extract solutions incubated with reaction mixtures A, B, and C were pipetted into small test tubes and injected with 0.1 ml of the firefly extract solution. This solution was kept cool on ice throughout the assay. The peak height of the emitted light was measured on an Aminco Chem-Glo Photometer.

ATP concentrations were calculated from a standard curve of freshly prepared ATP solutions and were based on the peak of emitted light produced by each of the 3 axis extract-reaction mixture solutions. The ATP content was determined from the extract solution containing reaction mixture A. The concentration of ADP was the difference between ATP concentrations in extracts containing reaction mixtures B and A. The AMP content was calculated by the ATP difference in extracts of reaction mixtures C and B.

Adenosine phosphate concentrations were expressed as nmoles per axis with values entered into the following equation of Atkinson (1968) to derive the energy charge:

$$\text{ENERGY CHARGE} = \frac{(\text{ATP}) + 0.5\ (\text{ADP})}{(\text{ATP}) + (\text{ADP}) + (\text{AMP})}$$

Experiment 3. Compositional Changes in Metabolic Reserve Materials

Replicated groups of 50 aged and nonaged seeds were imbibed or germinated at 25^{o} C for specific times using similar methodology as described in Chapter II. At 0, 24, 48, 72, and 96 hours, seeds or seedlings were separated into axes and cotyledons after germination

counts were made. The dissected seed parts were kept cool on ice while average axis lengths were determined. Axis and cotyledon fresh weights were measured and the samples were quickly frozen at -40° C. All seedling material, including axes and cotyledons from unimbibed seeds, were freeze-dried for a minimum of 48 hours. Dry weights were taken on the lypholized material.

The dried axes and cotyledons were ground to fine powders with a Virtis '45' homogenizer (90 seconds at setting 40) prior to analyzing for total quantities of soluble sugars, soluble reducing sugars, protein, and amino acids.

Soluble sugars in the axes were extracted from 100 mg quantities of the ground material by the addition of 5 ml of hot 80% (volume/volume) ethanol (Adams et al., 1980). The mixtures were heated at 75° C for 15 minutes, then centrifuged at 15,000 x g for 10 minutes. The supernatant solutions containing the soluble sugars were removed and the residues re-extracted 2 additional times in the same manner using 2.5 ml volumes of hot 80% ethanol. The supernatant fractions were combined with the original supernatant.

Cotyledon soluble sugars were extracted using the same procedure as the axes with slight modifications. Sample amounts and the initial volume of ethanol were increased to 400 mg and 20 ml, respectively. The 2 subsequent extractions were each made with 10 ml of 80% ethanol.

Total soluble sugar content in the extracts was determined colormetrically by the phenol-sulfuric acid method as described by Hodge and Hefreitor (1962), using glucose standards prepared in 80% ethanol. Absorbances were read at 490 nm. Values obtained from the standard

curve were expressed as mg total soluble sugars per 100 mg dry axis or cotyledon weight.

Another 1 ml aliquot was removed from the sugar extract solutions for the colormetric determination of axis and cotyledon reducing sugars following the procedure of Somogyi (1952). Glucose in 80% ethanol was again used in the preparation of the standard curve. Absorbances of the solutions were read at 500 nm with values calculated from the standard curve as mg of reducing sugars per 100 mg dry axis or cotyledon weight.

One hundred milligram quantities of ground axis or cotyledon material were used to determine protein and amino acid content as described by Cruz et al. (1970). Sample powders were each suspended in 5 ml of 10% (volume/volume) trichloroacetic acid (TCA) and held in ice for 2 hours to percipitate the protein. The solutions were centrifuged at 1200 x g at 4^{o} C for 10 minutes. After decanting the supernatant solution containing the free amino acids, the pellet was re-extracted 2 more times in the same manner using 2.5 ml volumes of ice-cold 10% TCA. The additional supernatant fractions were combined with the original fraction after centrifugation and saved for amino acid analysis.

Proteins remaining in the pellet were suspended in 10 ml of 0.5 N NaOH, vortexed, and dissolved by heating at 80^{o} C for 10 minutes. After cooling, cellular debris was removed from the solubolized protein solutions by centrifugation at 9,000 x g for 15 minutes. A 1 ml portion of the supernatant was taken for protein determination by the method of Lowrey et al. (1951) as described by Cooper (1977). When necessary, the protein solutions were diluted with additional 0.5 N NaOH before assaying. Protein standards were prepared from bovine serum albumin dissolved in 0.5 N NaOH with absorbances of all solutions read at 540 nm.

Protein contents of the axes or cotyledons were determined from the standard curve and expressed as mg protein per 100 mg dry weight.

Total amino acids in the TCA fractions were analyzed by the method of Moore (1968). To 0.5 ml of the TCA solutions containing the amino acids was added 16.2 mg of Na_2CO_3 and 1 ml of ninhydrin reagent. Test tubes containing this mixture were covered with glass marbles and heated in boiling water for 15 minutes. When cool, the solutions were diluted 10 fold with 50% (volume/volume) ethanol and vortexed. Absorbances of the solutions were then read at 570 nm. The standard used was L-leucine dissolved in 10% TCA. Values determined from the standard curve were expressed as mg of amino acids per 100 mg dry axis or cotyledon weight.

Experiment 4. Changes in Protein Synthesis of the Seedling Axes

Replicated samples of 5 aged and nonaged seeds were imbibed or germinated at 25° C for 10, 22, 44, 70, and 94 hours using similar methodology as described in Chapter II. At the end of each time period, germination counts were taken on 4 samples per treatment. The seeds or seedlings were decoated by hand and the average axis length of each sample was determined. The procedure outlined by Abdul-Baki (1969b) was then used as the basis for measuring axis incorporation of radio-labelled leucine into proteins.

Seeds or seedlings were placed in large glass test tubes with 2.5 ml of 10^{-4} M leucine containing 1 μc per ml (U-^{14}C) - leucine (Shwarz Mann Co.). The test tubes were sealed and slowly rotated in a horizontal position in a tube roller for 2 hours at 25° C in darkness. Seed tissue was constantly bathed but not submerged in the ^{14}C-labelling medium. Immediately after the incubation period, seeds or seedlings

were transferred into ice and washed 4 times with 25 ml of ice-cold water. Axes from each sample were then separated from the cotyledons, washed 2 additional times with 50 ml of ice-cold distilled water, frozen at -40° C, and freeze-dried for 48 hours. The dried axis samples were ground to fine powders in a mortar after weighing.

To remove the unincorporated ^{14}C-leucine from the tissue, a maximum of 30 mg of the axis powder from each sample was vortexed with 3 ml of ice-cold 10% (volume/volume) TCA in a small centrifuge tube. The solutions were held at 0° C for 2 hours with additional mixing approximately every 15 minutes. After centrifugation at 9,000 x g and 4° C for 15 minutes, the supernatant fractions containing the unincorporated label were decanted. The pellets were re-extracted 2 more times in the same manner with 1 ml volumes of 10% TCA which were added to the original supernatant fractions.

Precipitated proteins remaining in the pellets were dissolved in 2.5 ml of 0.5 N NaOH by mixing and heating at 80° C for 15 minutes. Insoluble material was removed by centrifugation at 9,000 x g for 10 minutes at 20° C, with the supernatant referred to as the protein fraction.

For each sample, 1 ml aliquots of the unincorporated ^{14}C-leucine fractions and the soluble protein fractions were placed into separate 20 ml glass scintillation vials. Nineteen milliliters of Aquasol scintillation solution was then added to each vial and mixed. The samples were counted on a Beckman Model LS 7500 liquid scintillation counter. All values were corrected by external standard method to disintegrations per minute (dpm) per mg dry axis weight (Wang et al., 1975). Counting efficiencies were 41% and 55% for the unincorporated ^{14}C-leucine and

the protein fractions, respectively. Total uptake of ^{14}C-leucine per mg dry axis weight was calculated by summing the values of each of the 2 fractions.

Experiment 5. Initiation of Root Tip Cell Division

Replicated samples of 50 aged and nonaged seeds were imbibed or germinated at 25° C for 24, 48, 72, and 96 hours using similar methodology as described in Chapter II. At the end of each time period germination counts were taken on 4 samples per treatment. Axis lengths were measured after seed coat removal. Three millimeter root tip sections were removed with a scalpel from 10 randomly selected seedlings per sample. The root tips were then placed in small vials containing 5 ml of Carnoy's fixative (Dyer, 1963). The vials were kept refrigerated at approximately 7° C until further use. Axes were removed from the remaining 40 seedlings of each sample and weighed. Axis dry weights were recorded after oven-drying at 70° C for 48 hours.

Squash mounts for identifying actively dividing cells in the root tips were prepared according to the method of Dyer (1963). For each 24 hour imbibition period a random sample of 10 root tips from aged and nonaged seedlings were selected from the fixative solution. Each root tip then represented a replication. The root tips were hydrolized in several milliliters of a 45% (volume/volume) acetic acid solution for 10 minutes and individually transferred to a clean glass microscope slide. A small quantity of the 45% acetic acid solution was added to the material to prevent drying. Cells of the root tip were then separated with needles. Excess acetic acid was blotted away with tissue paper. Chromosomes of actively dividing cells were stained by the addition of lacto-propionic

orcein (Dyer, 1963). Staining of the chromosomes was continued for at least 1 hour by placing the slides in a closed chamber saturated with the 45% acetic acid solution. Upon removal from the chamber, a glass cover slip was placed over the cells and tapped with the point of a needle to remove air bubbles and spread the cells. The slides were then heated, without boiling, for a few seconds. Tissue paper was placed over the cover slip and firmly pressed to flatten the cells and remove any excess stain. The edges of the cover slip were sealed with clear fingernail polish.

To detect the onset of cell division and determine mitotic activity, 100 cells per root preparation were examined using a bright field microscope. A cell was counted as actively dividing if chromosomes could be observed. Data recorded for each treatment at each sample time included the percent of root tips exhibiting cell division and the percent of total root cells dividing.

Results and Discussion

Experiment 1. Respiratory Activity Changes

Accelerated aging led to reduced initial respiratory activity of the axes, but not the cotyledons of soybean (Figures IV-1, 2). Leopold and Musgrave (1980) also found that the adverse effects of accelerated aging on respiratory activity were more pronounced in the axes than in the cotyledons after 1 hour of hydration, although measurements were made on seed part fragments of soybeans.

Respiration of axes from aged seeds increased 10 fold during the subsequent 24 hours of imbibition but was only half the rate of nonaged axes (Figure IV-1). There was no appreciable difference in axis dry weight at 24 hours, but nonaged axes had significantly greater lengths

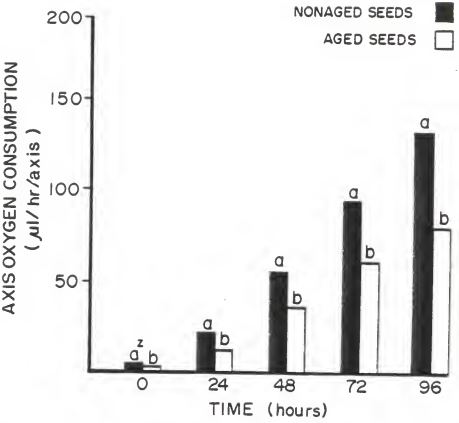

Figure IV-1. Axis respiratory activity of nonaged and accelerated aged soybean seeds or seedlings determined by oxygen consumption on a per axis basis.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

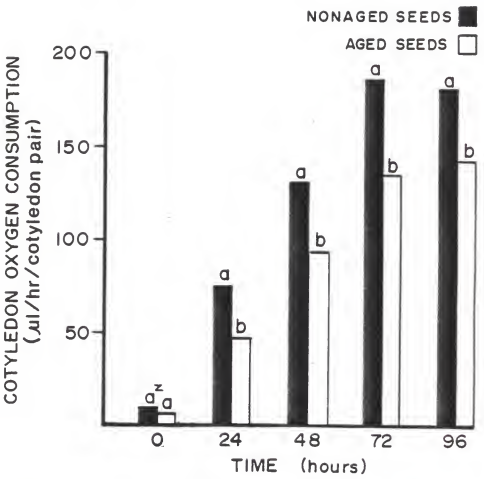

Figure IV-2. Cotyledon respiratory activity of nonaged and accelerated aged soybean seeds or seedlings determined by oxygen consumption on a per cotyledon pair basis.

[z] Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

and fresh weights (Table IV-1). When calculated on an individual axis basis, oxygen uptake values indicated that nonaged axis respiratory activity continually exceeded that of aged axes throughout the final 72 hours of germination and seedling growth (Figure IV-1). Wahab and Burris (1971) reported similar trends in respiratory activity between high and low vigor soybean seeds germinated for 72 hours. In their study low vigor seeds were from a lot which was stored for 2 years under ambient climatic conditions in Iowa, whereas high vigor seeds were stored at the same location for only 1 year and maintained at 10^{o} C and 4.7% moisture. In their study and in the present investigation differences in respiratory activity, when expressed on a per axis basis, can be primarily attributed to the larger size of the high vigor or nonaged axes. According to Burris et al. (1971), the higher respiratory activity may reflect a greater rate of cell division during the later stages of early seedling growth.

Determining oxygen consumption as a function of axis dry weight, however, revealed that the respiratory rate of aged axes generally exceeded that of nonaged axes from 48 to 96 hours (Figure IV-3). In light of previous research, the data presented in this form may be questionable. Burris et al. (1971) noted that soybean axis oxygen consumption determined on a dry weight basis decreased when structural material, such as cell wall polysaccharides, began to occupy a greater proportion of the axis dry weight. In the present experiment nonaged axes had substantial dry weight accumulation by 48 hours, while no appreciable dry weight increase was found in aged axes until 72 hours (Table IV-1). Thus, when based on axis dry weight, oxygen consumption values may have

Table IV-1. Seedling growth measurements of nonaged and accelerated aged soybean seeds used in respiratory activity determinations.

Seed Treatment	Germination Time (hours)				
	0	24	48	72	96
			Germination Percent		
Nonaged	0	45	100	100	100
Aged	0	12	100	100	100
	ns[z]	*	ns	ns	ns
			Axis Fresh Weight (mg/axis)		
Nonaged	11.4	17.9	92.5	221.6	415.4
Aged	10.0	12.9	30.4	109.7	197.7
	*	*	*	*	*
			Axis Dry Weight (mg/axis)		
Nonaged	4.2	4.4	8.5	17.3	28.7
Aged	3.8	4.1	4.4	9.5	14.3
	ns	ns	*	*	*
			Axis Length (mm/axis)		
Nonaged	4.0	9.5	85.6	86.4	155.9
Aged	4.0	7.3	14.3	40.2	75.1
	ns	*	*	*	*
			Cotyledon Fresh Weight (mg/cotyledon pair)		
Nonaged	242.9	351.0	335.6	348.1	336.1
Aged	227.7	320.7	339.9	332.6	343.5
	ns	ns	ns	ns	ns
			Cotyledon Dry Weight (mg/cotyledon pair)		
Nonaged	146.8	147.6	133.3	126.5	118.3
Aged	134.6	134.2	138.2	128.6	123.3
	*	ns	ns	ns	ns

[z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant at 5% level.

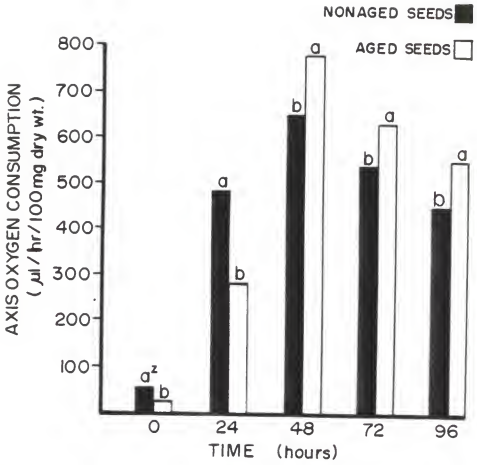

Figure IV-3. Axis respiratory activity of nonaged and accelerated aged soybean seeds or seedlings determined by oxygen consumption on a dry weight basis.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

been biased simply because structural material comprised a greater proportion of the axis dry weight in nonaged seeds compared to aged seeds.

In another relevant study, Abu-Shakra and Ching (1967) isolated equal quantities of mitochondria from 4-day-old seedling axes of high and low vigor soybeans. They found a greater rate of oxygen uptake but lower phosphorylative activity in the low vigor material. Based on these results, it can be seen that an increased rate of oxygen consumption does not necessarily denote greater respiratory efficiency.

From 24 to 96 hours a lower respiratory rate was observed in the cotyledons previously exposed to accelerated aging conditions. This lower rate of oxygen uptake in aged cotyledons was apparent when values were calculated on a dry weight (Figure IV-4) as well as a per seed basis (Figure IV-2). Wahab and Burris (1971) found that deterioration of soybean seed vigor resulting from prolonged aging also impaired respiratory activity of the cotyledons throughout 72 hours of seedling development.

Experiment 2. Changes in Axis Energy Charge and Axis Phosphorylated Adenylates

Energy charge is defined as half the average number of anhydride-bound phosphate groups per adenine moiety, and has been proposed as a measurement of energy state regulation of various biological systems (Atkinson, 1968). It has been postulated that when energy charge values exceed 0.5, ATP-utilizing systems or biosynthetic systems become active. When the energy charge is less than 0.5, ATP-regenerating systems are predominately active (Ching, 1973).

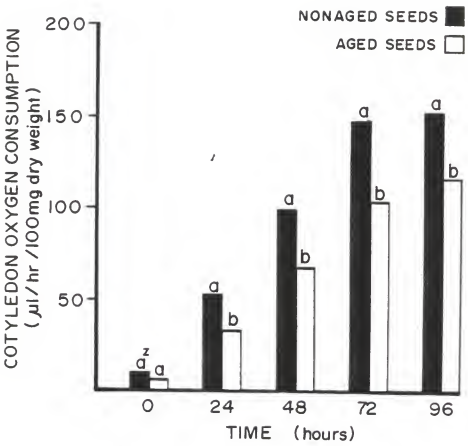

Figure IV-4. Cotyledon respiratory activity of nonaged and accelerated aged soybean seeds or seedlings determined by oxygen consumption on a dry weight basis.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

Energy charge was higher in the axes of accelerated aged seeds (0.34) compared to the axes of nonaged seeds (0.21) before imbibition had begun (Figure IV-5). This could be accounted for by a greater ADP concentration in the aged axes, since axis ATP and AMP levels did not differ between aged and nonaged seeds (Figures IV-6, 7, 9). There was a slight increase in axis length (Table IV-2) and axis energy charge (Figure IV-5) in the aged seeds after 24 hours of imbibition. At the same time a greater increase in nonaged axis length was noted (Table IV-2), but no change in energy charge was observed (Figure IV-5). At 24 hours the ATP content was the same as that in aged axes (Figure IV-6), but a larger quantity of axis AMP in nonaged seeds led to the absence of an equivalent rise in the nonaged axis energy charge (Figure IV-5).

The concentrations of ATP and ADP varied with time (Figures IV-6, 7), and followed no consistent patterns that related to the differences in seedling growth between aged and nonaged seeds during the last 72 hours of growth (Table IV-2). Higher amounts of total phosphorylated adenylates were generally found in the nonaged axes compared to the aged axes (Figure IV-9). This indicated that AMP synthesis might have been impaired by accelerated aging (Figure IV-8). A more substantial increase in axis dry weight was observed in aged seeds as total phosphorylated adenylates increased in these axes during the final 48 hour period of growth.

Axis growth, as measured by fresh weight and length was greater in nonaged than aged throughout the majority of the 96 hour germination period (Table IV-2). Despite this dissimilarity in axis growth, very little or no difference in the adenylate energy state between aged and nonaged axes could be discerned solely on the basis of energy charge.

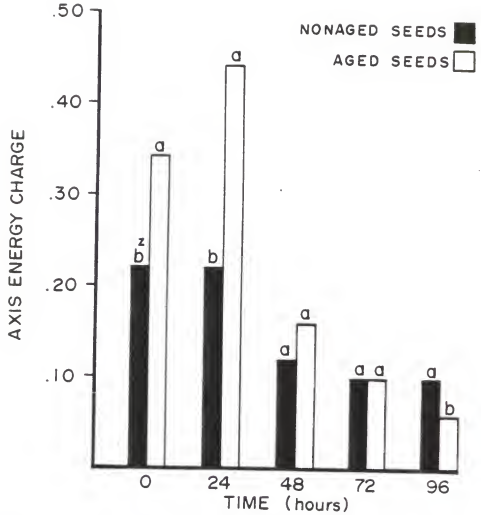

Figure IV-5. Axis energy charge values of nonaged and accelerated aged soybean seeds or seedlings.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

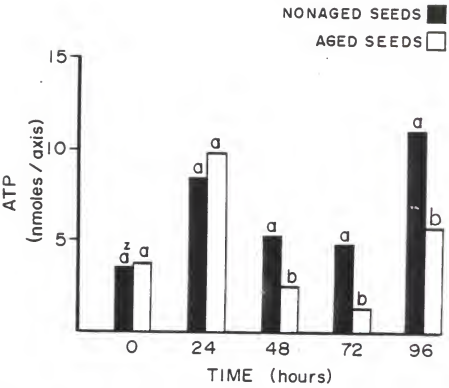

Figure IV-6. Axis ATP content of nonaged and accelerated aged soybean seeds or seedlings.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

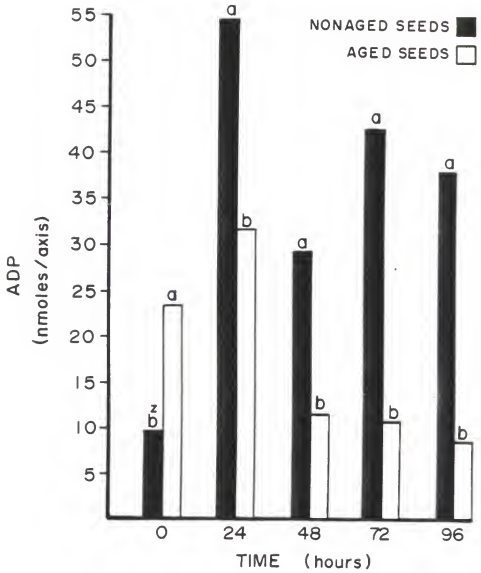

Figure IV-7. Axis ADP content of nonaged and accelerated aged soybean seeds or seedlings.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

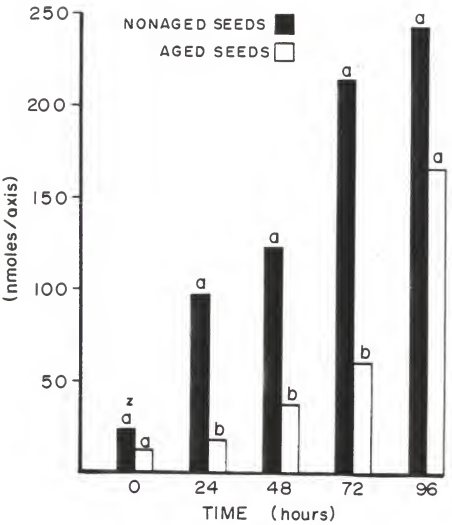

Figure IV-8. Axis AMP content of nonaged and accelerated aged soybean seeds or seedlings.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

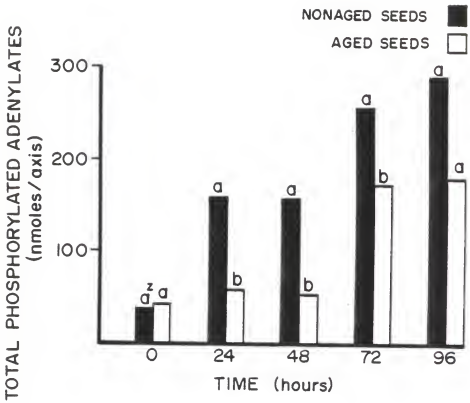

Figure IV-9. Axis total phosphorylated adenylate content of nonaged and accelerated aged soybean seeds or seedlings.

[z] Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

Table IV-2. Seedling growth measurements of nonaged and accelerated aged soybean seeds used in axis energy charge and axis phosphorylated adenylated content determinations

Seed Treatment	Germination Time (hours)				
	0	24	48	72	96
			Germination Percent		
Nonaged	0	40	96	98	98
Aged	0	10	75	96	98
	ns[z]	*	*	ns	ns
			Axis Fresh Weight (mg/axis)		
Nonaged	4.6	19.1	42.6	115.4	269.5
Aged	4.8	18.9	23.5	66.4	152.7
	ns	ns	*	*	*
			Axis Length (mm/axis)		
Nonaged	4.0	10.2	23.1	71.4	137.7
Aged	4.0	5.5	9.6	31.6	46.2
	ns	*	*	*	*

[z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant at 5% level.

Anderson (1977) was able to directly correlate soybean seed vigor deterioration induced by accelerated aging to a reduction in axis energy charge. However, determinations were made after only 1.5 or 3 hours of imbibition. In his study aged seeds from which energy charge measurements were made had a much lower germination percentage than the nonaged group. Thus, energy charge values may have reflected viability rather than vigor differences.

Based on the results of the present study, it appeared that during the later stages of germination energy charge evaluations were not a key factor related to differential seedling growth between aged and nonaged seeds (Figure IV-5). Energy charge only takes into account the relative proportions of ATP, ADP, and AMP that are present in the tissue at a particular point in time. Although it can only be speculated, a slower rate of ATP turnover in the aged axes due to a reduced energy demand, supply, or a combination of the 2 may have led to a slower rate of seedling growth.

Aged and nonaged seeds had low axis energy charge values (less than 0.2) during the 24 to 48 hour period of germination (Figure IV-5). Values were below this level for the remainder of the germination period. According to Ching's (1972b) explanation of the energy charge concept, these observations suggested that the activity of ATP-regenerating processes far exceeded that of ATP-utilizing processes in the axes.

Independent of the accelerated aging treatment, the energy charge values found for developing soybean seed axes in this study were much lower than those reported for other types of metabolically active seeds. Crompton et al. (1978) found that peanut seeds maintained

energy charge values of approximately 0.9 through 48 hours of germination. During stratification, germination, and seedling development, pine seed embryo energy charges fluctuated but remained above 0.6 (Ching and Ching, 1972). High energy charge values have also been reported for germinating radish (Moreland et al., 1974), wheat (Ching and Kronstad, 1972), and barley seeds (Ching et al., 1977). Nevertheless, low energy charge values may not be unrealistic of actively growing seed tissue. Atkinson (1968, p. 4033) stated that "the metabolic sequences that lead to regeneration of ATP contain most of the primary intermediates that are used in biosynthesis. Thus replenishment of the pools of these intermediates cannot be separated from ATP regeneration." He added that "regulation by the energy charge alone would have the consequence that replenishment of primary biosynthetic intermediates would be severely limited when the energy charge was high. Thus a plentiful energy supply would depress biosynthetic activity." The low energy charge values determined in the present study may then have been a necessary requirement for axis growth irrespective of the rate at which it occurred.

Experiment 3. Compositional Changes in Metabolic Reserve Materials

The quantities of total soluble sugars were essentially the same in aged and nonaged seeds prior to imbibition, and accounted for approximately 21% and 12% of the axis and cotyledon dry weights, respectively (Figures IV-10, 11). The accelerated aging treatment had no significant effect on the initial reducing sugar contents of the cotyledons and axes (Figures IV-12, 13). In both tissues, reducing sugars comprised a relatively small portion of the dry seed weight compared to the total amount of sugars present. When Edje and Burris

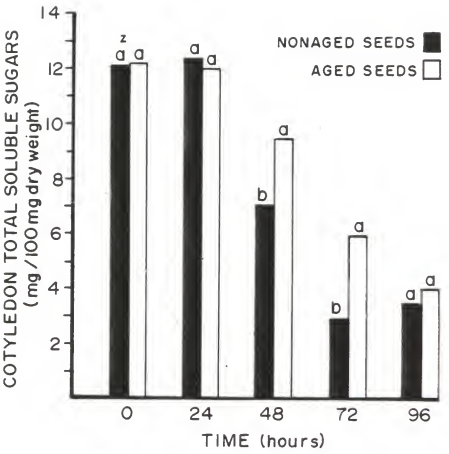

Figure IV-10. Cotyledon total soluble sugar content of nonaged and accelerated aged soybean seeds or seedlings.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

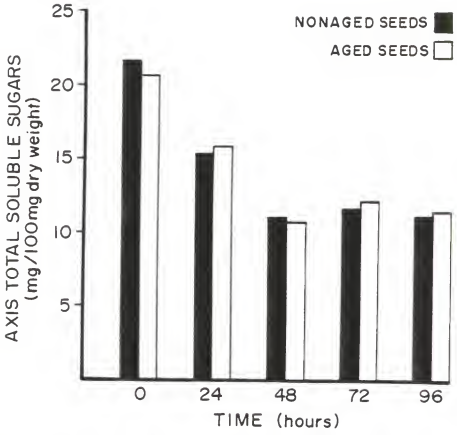

Figure IV-11. Axis total soluble sugar content of nonaged and accelerated aged soybean seeds or seedlings.

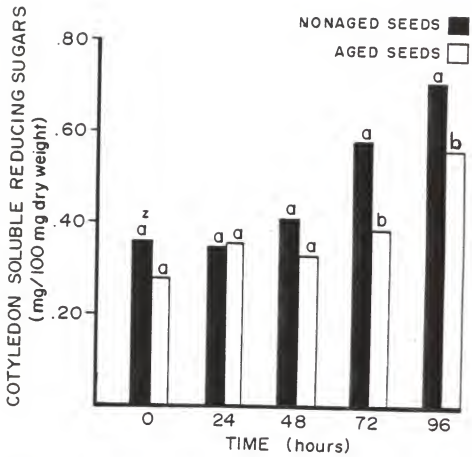

Figure IV-12. Cotyledon soluble reducing sugar content of nonaged and accelerated aged soybean seeds and seedlings.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

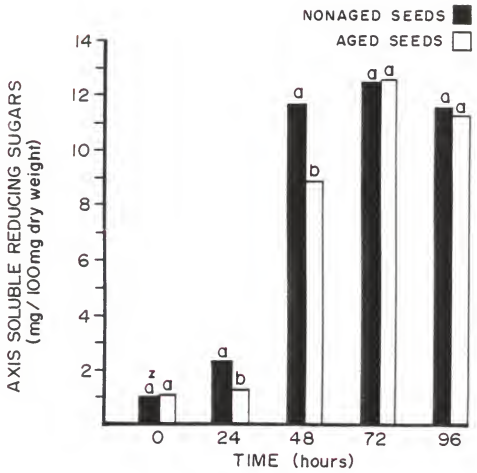

Figure IV-13. Axis soluble reducing sugar content of nonaged and accelerated aged soybean seeds or seedlings.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

(1970) aged soybean seeds for 4 days at 40° C and 13% moisture, no change in total or reducing sugars was detected in the unimbibed seeds. Longer periods of aging did result in progressive decline, which, according to the authors, might have been brought about by the activation of hydrolytic enzymes during the extended aging treatments.

There was very little change in total soluble sugar content in the cotyledons of aged and nonaged seeds for the first 24 hours of imbibition (Figure IV-10). During this time axis dry weights did not increase (Table IV-3.). From 24 to 48 hours soluble sugars in the cotyledons decreased, but to a greater extent in the nonaged cotyledons (Figure IV-10). A significantly higher dry weight was also observed for axes of nonaged seeds at 48 hours (Table IV-2), suggesting that mobilization of carbohydrates and/or their utilization for seedling growth may have been impaired by the aging treatment. A similar relationship between soluble sugars in the cotyledons and axis dry weights was still evident at 72 hours (Figure IV-10, Table IV-3). Aged axes had approximately half the dry weight of nonaged axes but twice the amount of soluble sugars in the cotyledons. By 96 hours there was no difference in the quantity of soluble sugars present in the aged and nonaged cotyledons (Figure IV-10); however aged axis dry weights and axis lengths were still considerably below those of the nonaged seedlings (Table IV-3).

There was no appreciable change in the content of cotyledon reducing sugars of aged and nonaged seeds during the first 24 hours of imbibition (Figure IV-12). After 24 hours of imbibition, nonaged cotyledons showed a progressive increase in the amount of reducing sugars. In contrast, aged cotyledon reducing sugar content fluctuated

Table IV-3. Seedling growth measurements of nonaged and accelerated aged soybean seeds used in metabolic reserve material determinations.

Seed Treatment	Germination Time (hours) 0	24	48	72	96
			Germination Percent		
Nonaged	0	35	98	100	100
Aged	0	12	84	100	100
	ns[z]	*	*	ns	ns
			Axis Fresh Weight (mg/axis)		
Nonaged	4.4	12.1	65.0	238.0	377.7
Aged	4.4	10.6	21.3	77.6	158.6
	ns	ns	*	*	*
			Axis Dry Weight (mg/axis)		
Nonaged	4.3	4.3	9.5	18.3	29.2
Aged	4.2	4.1	5.2	9.9	16.0
	ns	ns	*	*	*
			Axis Length (mm/axis)		
Nonaged	4.0	8.2	30.0	100.8	142.3
Aged	4.0	7.0	12.2	30.5	54.6
	ns	ns	*	*	*
			Cotyledon Fresh Weight (mg/cotyledon pair)		
Nonaged	167.1	325.0	323.6	338.9	354.6
Aged	158.9	317.4	324.9	321.9	337.9
	ns	ns	ns	ns	ns
			Cotyledon Dry Weight (mg/cotyledon pair)		
Nonaged	155.8	142.0	135.3	125.4	111.7
Aged	147.8	137.2	136.7	127.6	126.1
	*	*	*	*	*

[z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant at 5% level.

with no definite increase occurring until 96 hours. Statistically significant differences were not consistently observed, but there appeared to be a trend toward lower levels of reducing sugars in aged than nonaged cotyledons during the latter half of the germination period. Previous research has identified that reducing sugars in soybean cotyledons accumulate primarily as glucose and fructose from the breakdown of nonreducing polysaccharides, specifically stachyose, raffinose, and sucrose (Pazur et al., 1962). Therefore, the lower reducing sugar content in the aged cotyledons may have been a limiting factor preventing optimal respiratory activity as reported previously (Figures IV-1, 3).

When determined on a dry weight basis there were no differences in total soluble sugar content between the axes of aged and nonaged seeds throughout the 96 hour germination period (Figure IV-11). The highest quantities of axis soluble sugars were present in the unimbibed seeds, but once imbibition began levels fell in both seed types until 48 hours. Thus, the majority of the carbohydrates utilized by the axis during this period most likely originated from the axis itself. There appeared to be less efficient utilization of these sugars in the axes of aged seeds as evidenced by several factors associated with vigorous seedling growth. At 24 hours, fewer aged seeds had germinated and the axis lengths of these seeds were somewhat shorter compared to the nonaged group (Table IV-3). In addition, significantly greater axis dry weights in nonaged seeds became evident by 48 hours.

After 48 hours total soluble sugars in the aged and nonaged axes remained relatively constant (Figure IV-11). This suggested that the soluble sugars present in the axes were then being supplied by the

cotyledons. Here again, carbohydrate transport from the cotyledons to the axes seemed to be adversely affected by accelerated aging, as the dry weight of nonaged axes was approximately twice that of the aged axes after 48 hours of germination (Table IV-3).

Although the accelerated aging treatment did not affect the initial level of soluble reducing sugars in the axes, higher quantities were observed in the nonaged axes for the first 48 hours of germination (Figure IV-13). Nonaged axes also showed greater increases in length and fresh weight during this time and by 48 hours had attained greater dry weight (Table IV-3). Apparently, initial axis growth was associated more closely with the amount of reducing sugars rather than the total quantity of soluble sugars present in the axis. Once axis reducing sugars reached high levels, axis dry weights and lengths increased in both nonaged and aged seeds (Figure IV-13, Table IV-3).

Accelerated aging had no apparent influence on the initial protein content of the cotyledons (Figure IV-14). Once imbibition had begun, no differences in cotyledon protein content between aged and nonaged seeds were observed through 96 hours of germination (Figure IV-14). Because cotyledon dry weight differences were not consistently observed between aged and nonaged seeds, it was not possible to discern any definite relationship between cotyledon protein content and seed vigor expression (Table IV-3).

The initial content of axis protein was also unaffected by the accelerated aging treatment (Figure IV-15). Quantities remained relatively unchanged for the first 24 hours of imbibition. As dry weight accumulation became evident in nonaged axes at 48 hours, protein content steadily decreased. A similar relationship between axis dry weight and

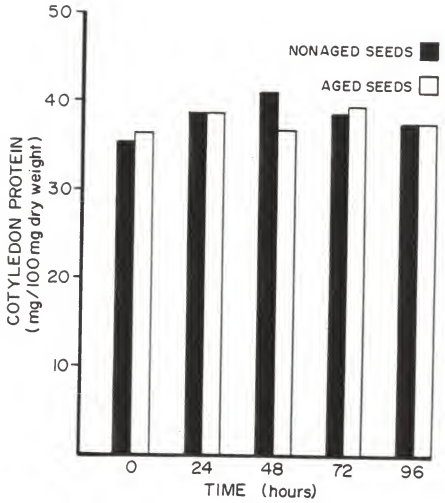

Figure IV-14. Cotyledon protein content of nonaged and accelerated aged sobyean seeds or seedlings.

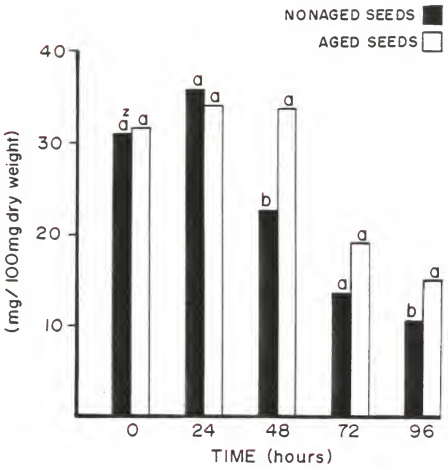

Figure IV-15. Axis protein content of nonaged and accelerated aged soybean seeds or seedlings.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

axis protein content was observed in aged axes, but these changes lagged 24 hours behind nonaged axes, as aged axes did not show a substantial dry weight increase until 72 hours (Table IV-3).

A slightly, but significantly, greater amount of amino acids was found in the cotyledons of aged seeds prior to imbibition (Figure IV-16). This difference was not large enough to have been reflected by a reduction in aged cotyledon protein content (Figure IV-14). However, this observation did suggest that enzymatic hydrolysis of protein reserves may have occurred during the accelerated aging treatment. Perl et al. (1978) demonstrated that protease activity was promoted during accelerated aging of sorghum seeds. In addition to finding higher amino acid levels, they noted decreased activity of several other enzymes following accelerated aging. These included amylase, acid phosphatase, RNase, and glutmate transaminase. The authors postulated that protease may have caused the drop in activity of these enzymes which consequently led to the loss of seed vigor. Under prolonged aging conditions, Ching and Schoolcraft (1968) reported an increase in free amino acids occurred in crimson clover and ryegrass seeds as storage temperature and seed moisture increased. Halder and Gupta (1980) noted similar results in sunflower seeds but were unable to detect any appreciable change in protein content.

Once the seeds had begun to imbibe water, amino acid content in the cotyledons increased, indicating that storage proteins were being degraded (Figure IV-16). The initial difference in cotyledon amino acid content due to accelerated aging was no longer evident by 24 hours. The rate of protein hydrolysis apparently exceeded the rate of amino acid mobilization and utilization as levels of amino acids in both

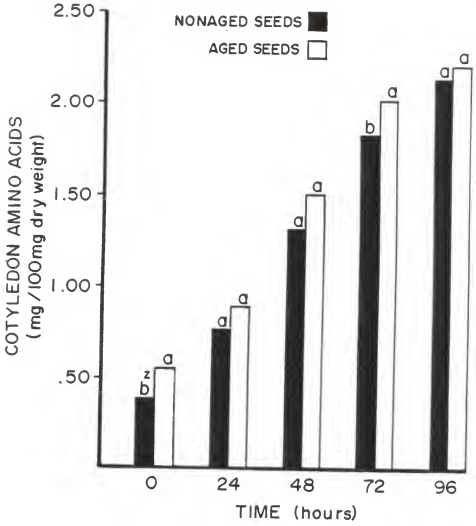

Figure IV-16. Cotyledon amino acid content of nonaged and accelerated aged soybean seeds or seedlings.

[z]Mean separation between treatment at each time by Duncan's multiple range test, 5% level.

seed types progressively increased during the remaining 72 hours of the test. Only minor, if any, quantitative differences were noted during this time despite the fact that seedlings from aged seeds had consistent slower growth (Table IV-3). In naturally aged soybean seeds, Wahab and Burris (1971) found that vigor deterioration was associated with reduced quantities of amino acids in the cotyledons, but only after 48 hours of germination.

Accelerated aging did not apparently alter the amount of amino acids in the axes of dry seeds (Figure IV-17). The lowest levels of amino acids were found prior to imbibition. An equivalent rise in amino acid content was observed in aged and nonaged axes within the first 24 hours of imbibition. During this time neither aged nor nonaged axis dry weight increased (Table IV-3). A substantial increase of amino acids was evident in nonaged axes by 48 hours, while the amount of aged axes declined to a level approximately equal to that found in the dry seed (Figure IV-17). The rise in amino acid content in the axes of nonaged seeds closely corresponded to increased axis dry weight and axis length of these seeds, whereas only minor dry weight accumulation had occurred in the axes of aged seeds by 48 hours (Table IV-3). These results are similar to those reported by Wahab and Burris (1971) for naturally aged soybean seeds where low amino acid concentrations were found during the early stages of germination. But contrary to their results with naturally aged soybean seeds, accelerated aging did not permanently limit the build up of axis amino acids. After 48 hours, levels steadily increased in the aged axes, although quantities remained lower than in nonaged axes (Figure IV-17). Associated with the larger

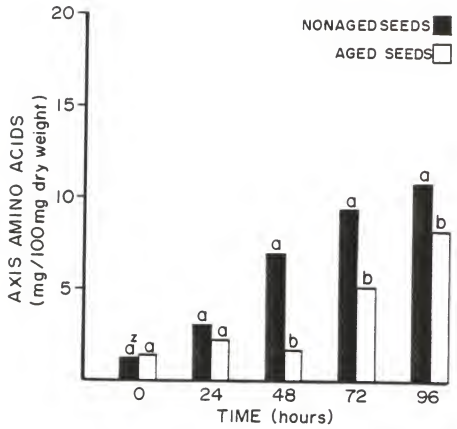

Figure IV-17. Axis amino acid content of nonaged and accelerated aged soybean seeds or seedlings.

[z]Mean separation between treatment at each time by Duncan's multiple range test, 5% level.

amino acid pool size in the aged axes was a greater rate of seedling growth by 72 hours as measured by axis dry weight and axis length (Table IV-3).

Based on the results from both aged and nonaged seeds, it was apparent that a sufficient supply of amino acids must be present in the axis for seedling growth to take place. The lower level of amino acids in the aged seed axes may have adversely affected several different biochemical processes essential to vigorous seedling growth. This group of metabolities function not only as substrates for enzyme and structural protein synthesis, but also provide the carbon skeletons for Kreb's cycle intermediates and nucleic acid synthesis (Lehninger, 1978).

Experiment 4. Changes in Protein Synthesis of the Seedling Axes

The initial capacity for protein synthesis in soybean axes during the first 24 hours of germination was not completely inhibited, but appeared to be reduced by the accelerated aging treatment (Table IV-4). There were no differences in axis dry weights between treatments during this time, but nonaged axes had elongated to a greater extent than aged axes by 24 hours (Table IV-5). Consequently, a greater germination percentage was observed in the nonaged seeds at this time.

The impairment of protein synthesis may have been responsible for, or at least related to, the reduced increase in elongation of the aged axes. Klein et al. (1971) and others (Fujisawa, 1966; Walton and Soofi, 1969) demonstrated that protein synthesis in excised seed axes markedly increased during the first few hours of imbibition prior to cell elongation. When inhibitors, such as cycloheximide, were included in the imbibition medium, protein synthesis activity was almost completely absent and axis elongation failed to occur.

Table IV-4. Total uptake of ^{14}C-leucine and incorporation into protein (TCA precipitatable material) by axes of nonaged and accelerated aged soybean seeds or seedlings.

Seed Treatment	Germination Time (hours)				
	12	24	48	72	96
		Total Uptake of ^{14}C-Leucine (dpm/mg axis dry weight)			
Nonaged	776	1071	15777	16030	13684
Aged	614	741	5671	13405	13330
	ns[z]	ns	*	ns	ns
	^{14}C-Leucine Incorporated Into Protein (dpm/mg axis dry weight)				
Nonaged	216	366	5581	3446	3715
Aged	85	150	2630	4765	3926
	*	*	ns	ns	ns

[z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant at 5% level.

Table IV-5. Seedling growth measurements of nonaged and accelerated aged soybean seeds used in axis protein synthesis determinations.

Seed Treatment	Germination Time (hours)				
	12	24	48	72	96
		Germination Percent			
Nonaged	0	10	96	98	98
Aged	0	0	62	94	96
	ns[z]	*	*	*	ns
		Axis Dry Weight (mg/axis)			
Nonaged	3.8	4.7	6.5	14.1	22.3
Aged	4.0	4.1	4.3	7.4	11.6
	ns	ns	*	*	*
		Axis Length (mm/axis)			
Nonaged	4.7	8.4	22.0	70.4	139.1
Aged	4.8	6.4	10.4	26.1	50.9
	ns	*	*	*	*

[z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant at 5% level.

Several investigators have suggested that newly formed proteins in imbibing seeds are coded for by conserved messenger RNA and translated on ribosomes that have remained stable in the dry seed (Speigel and Marcus, 1975; Weeks and Marcus, 1971). Other research has pointed out that proteins may arise via transcription of new messenger RNA synthesized soon after the start of imbibition (Dobrzanska et al., 1973; Sen and Osborne, 1977). Therefore the reduction in labelled leucine incorporation into proteins in the aged soybean axes observed during the imbibition phase of germination may have been due to impairment of preformed or newly synthesized components of the protein synthesizing system. Pertinent literature is lacking for soybean seeds, but Bray and Chow (1976) demonstrated with pea seed axes that viability loss was accompanied by the breakdown of pre-existing ribosomal RNA soon after the start of imbibition. Roberts et al. (1973) reported similar findings in aged rye seeds. In both studies, ribosomes from these seed tissues had an impaired capacity for in vitro assembly of amino acids into polypeptide chains. In another cell-free study, Roberts and Osborne (1973) found that low vigor and reduced protein synthesis activity in 5-hour-imbibed rye seeds was associated with a decline in enzyme activity necessary for the binding of the transfer RNA-amino acid complex to the ribosomes. The decline in protein synthesis in seeds where vigor was reduced through natural or accelerated aging has also been related to a lower level of de novo synthesis of all classes of RNA (Ching, 1972a; Osborne et al., 1977; Van Onckelen et al., 1974). Impaired RNA synthesis has, in turn, been attributed to the alteration or degradation of DNA templates during seed aging (Cheah and Osborne, 1978).

Compared to previous levels, considerably greater amounts of labelled leucine were detected in the protein fractions of both aged and nonaged axes at 48 hours (Table IV-4). The value noted for nonaged seeds at this time indicated that protein synthesis was highly active. This peak in synthetic activity coincided with essentially complete germination of the nonaged seeds and the initiation of axis dry weight accumulation (Table IV-5). Maximum protein synthesis activity in aged axes was not attained until 24 hours later (Table IV-4) but was similarly associated with a high number of germinated seeds and an increase in axis dry weight (Table IV-5). The peaks observed in protein synthesis activity in both aged and nonaged axes closely corresponded to increased supplies of axis amino acids reported previously (Figure IV-17). Thus substrate availability may have been the primary factor preventing aged axes from reaching a maximum rate of protein production as readily as nonaged axes.

The detrimental effect of accelerated aging on axis protein synthesis was no longer evident by 96 hours. Equivalent amounts of ^{14}C-leucine were incorporated into the protein fractions of aged and nonaged axes on a dry weight basis (Table IV-4). This apparent recovery was correspondingly reflected by seedling growth (Table IV-5). Seedlings from both treatment groups proportionately increased axis dry weight and axis length by approximately 60% and 100%, respectively, during the final 24 hours of growth. Recovery of impaired metabolic processes in embryos from accelerated aged corn seeds has been reported by Berjak and Villiers (1972c). By cytological examination they were able to relate alterations in metabolic processes, including protein synthesis, to organelle damage. The damage was most noticeable shortly after the

beginning of imbibition but was found to be largely reversed by the time outward signs of radicle growth were visible. They suggested that a larger time taken for the aged seeds to germinate was brought about by the necessity for repair mechanisms to operate and restore metabolic function. A similar situation may have taken place in the aged soybean axes investigated in the present study.

Experiment 5. Initiation of Root Tip Cell Division

Neither aged or nonaged seeds exhibited root tip cell division by the end of the first 24 hours of imbibition (Table IV-6). Thus, the rate of cell elongation was the single factor accounting for the greater axis length and higher initial germination percent in the nonaged seeds at that time (Table IV-7). In most of the nonaged seeds radicle protrusion from the seed coat was accompanied by the initiation of cell division. By 48 hours cell division had commenced in the root tips of all nonaged seeds. Thereafter the mitotic rate steadily increased in the nonaged seedlings based on the percent of root cells dividing. These observations are in general agreement with a morphological study of soybeans by Miksche (1961). In his investigation mitotic figures in the radicle were observed 32 hours after the beginning of imbibition.

Aged axes continued to elongate and subsequently germinate between 24 and 48 hours (Table IV-7). However, cell division in the root tips was not observed until 72 hours (Table IV-6). Furthermore, when cell division was detected in the aged axes, the rate of activity was just slightly more than half that initially observed in the nonaged axes 24 hours earlier.

Accumulation of axis dry weight in both aged and nonaged seeds did not begin until the onset of cell division (Tables IV-6, 7).

Table IV-6. Root tip cell division activity of nonaged and accelerated aged soybean seeds or seedlings.

Seed Treatment	Germination Time (hours)			
	24	48	72	96
	Percent of Root Tips Exhibiting Division			
Nonaged	0	100	100	100
Aged	0	0	100	100
	Percent of Root Cells Dividing			
Nonaged	0	5.8	7.3	8.4
Aged	0	0	3.2	4.4
	ns[z]	*	*	*

[z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant at 5% level.

Table IV-7. Seedling growth measurements of nonaged and accelerated aged soybean seeds used in root tip cell division activity determinations.

Seed Treatment	Germination Time (hours) 24	48	72	96
		Germination Percent		
Nonaged	14	98	99	100
Aged	0	54	97	98
	*[Z]	*	ns	ns
		Axis Fresh Weight (mg/axis)		
Nonaged	11.8	44.0	156.8	284.2
Aged	10.5	14.8	44.9	108.7
	ns	*	*	*
		Axis Dry Weight (mg/axis)		
Nonaged	4.2	6.9	16.2	24.4
Aged	4.1	4.1	6.1	10.8
	ns	*	*	*
		Axis Length (mm/axis)		
Nonaged	8.2	27.5	74.4	143.7
Aged	6.9	9.6	25.1	49.5
	*	*	*	*

[Z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant at 5% level.

Previous studies have reported that accelerated aging of pea seeds (Murata et al., 1980) and natural aging of onion seeds (Orlova et al., 1975) resulted in a delay in mitotic activity. Moreover, subsequent cell division in these aged seeds were suppressed. Similar results were observed in the present study, as only a small increase in the percent of root tip cells dividing was evident in aged seedlings by 96 hours (Table IV-6).

From work conducted with barley seeds aged under high temperature and humidity stress, Murata et al. (1979) hypothesized that delay of the first mitosis and the slow activation of cell division may be related to the induction of chromosomal abnormalities during the aging process. They demonstrated that the frequency of such aberrations increased as the aging time increased. Abdalla and Roberts (1968) also identified chromosomal damage in the root tips of accelerated aged bean, barley, and pea seeds. They later determined that this injury eventually disappeared as seedling development proceeded and did not have a detrimental effect on yield (Abdalla and Roberts, 1969).

Summary

Several metabolic factors associated with energy production and utilization during germination and seedling development were investigated in relation to vigor deterioration of soybean seeds after accelerated aging. More specifically, respiratory activity, phosphorylated adenylate pool changes, protein synthesis, and early changes in metabolic reserve materials were examined and related to differences in the growth of aged and nonaged seeds.

Respiratory activity of aged seed axes was impaired during the first 24 hours after the start of imbibition. Thereafter, axis

respiration rates of aged seedlings exceeded those of nonaged seedlings. Despite the higher respiratory activity, aged axes consistently exhibited a slower rate of growth as measured by axis fresh and dry weights and axis lengths. Impaired respiratory activity in aged cotyledons was not apparent immediately after imbibition had begun but was observed throughout germination and seedling development.

No differences in axis energy charge levels were noted between aged and nonaged seeds during imbibition, germination, and seedling development. Changes in axis ATP and ADP contents fluctuated and followed no clear patterns that could be related to differences in seedling growth. However, the level of AMP and the total amount of phosphorylated adenylates were generally higher in the seedling axes of nonaged seeds compared to aged seeds.

Comparative measurements of several metabolic reserve materials in aged and nonaged seeds showed that the accelerated aging treatment had no effect on the initial amounts of total soluble sugars or soluble reducing sugars in the cotyledons and axes. On a dry weight basis, total soluble sugars decreased at equal rates in aged and nonaged axes during the first 48 hours after imbibition had been initiated and then remained fairly constant. Reducing sugars comprised a smaller proportion of the total soluble sugars in the aged axes than in nonaged axes until aged axis dry weight accumulation became evident. In the cotyledons of both aged and nonaged seeds the quantities of total soluble sugars showed no change for the first 24 hours but decreased more rapidly in nonaged cotyledons during the next 48 hours. Although significant differences were not consistantly observed, there was a

tendency for higher levels of reducing sugars to be maintained in nonaged cotyledons during the period of seedling development.

There were no differences in the protein content of unimbibed axes and cotyledons that could be attributed to the aging treatment. Axis protein content in imbibed aged and nonaged seeds declined as axis dry weight accumulated. Cotyledon protein content in aged and nonaged seeds decreased at approximately the same rate as the overall decrease in cotyledon dry weight. Because cotyledon dry weights were variable, it was not possible to distinguish a definite relationship between changes in cotyledon protein content and seed vigor expression.

A greater quantity of amino acids was detected in the cotyledons of unimbibed seeds following accelerated aging. However, this difference was no longer evident once the seeds were hydrated. Accelerated aging had no effect on the initial level of axis amino acids. A substantial increase in amino acids was found in nonaged axes 48 hours after the start of imbibition but was delayed an additional 24 hours in the aged axes. In both cases the rise in axis amino acid content corresponded to the first major increase in axis dry weight.

Axis protein synthesis in aged seeds was impaired for up to 72 hours relative to nonaged seeds. At that time protein synthesis activity in the aged axes peaked at a level comparable to that observed 24 hours earlier in nonaged axes. Here again, maximum rates of axis protein synthesis in both aged and nonaged seeds was associated with the initial accumulation of axis dry weight.

Cell division in the root tips of nonaged axes was first observed at 48 hours compared to 72 hours in aged axes. Moreover, the rate of

cell division in aged axes failed to reach a level equivalent to that of nonaged axes within the following 24 hours.

There appears to be no single metabolic factor which controls vigor deterioration in accelerated aged soybean seeds. Accelerated aging slows early seedling growth. However, respiratory activity, energy utilization, protein synthesis, and finally the cell division process appear to function, but slowly, in the aged seed axis. It is probable that accelerated aging leads to permanent damage in the seed at the molecular level during the aging process.

CHAPTER V
GROWTH AND RESPIRATORY METABOLISM OF EXCISED SOYBEAN SEED AXES IN RELATION TO ACCELERATED AGING AND EXTERNAL SUCROSE AVAILABILITY

The excision of unimbibed seed parts has been employed as a technique to more precisely isolate the site of metabolic disorders resulting from seed vigor deterioration. In several studies concerning vigor loss of soybean seeds, emphasis has been directed toward examining the metabolism of excised axes, since axis growth is often used as a primary criterion for evaluating seed vigor expression.

Anderson (1977) assayed RNA and protein synthesis in axes excised from dry soybean seeds in which different ranges of deterioration had been induced by high temperature and humidity stress. As the level of germinability declined, there was a corresponding decrease in the synthetic rates of these compounds after 3 hours of imbibition. The lower synthetic rates were correlated to reduced ATP content and lower energy charge in the deteriorated axes. Abdul-Baki and Chandra (1977) noted a reduction in total nucleic acid synthesis as well as protein synthesis when axes excised from accelerated aged soybean seeds were imbibed for 24 hours. Abdul-Baki and Anderson (1973) found that a decreased capacity to incorporate glucose into insoluble polysaccharides was associated with impaired respiratory activity of axes excised from deteriorated soybean seeds.

Leopold and Musgrave (1980) recently reported that accelerated aging of soybean seeds caused a marked decline in axis respiration,

mainly due to deterioration of the cytochrome pathway. Accompanying the deterioration of the cytochrome pathway was a shift in respiration to the less efficient alternate pathway in the aged axis. They suggested that energy (ATP) production would then be reduced even more than the lowered respiratory rate would indicate. Furthermore, they speculated that this consequence of impaired respiratory activity would limit biosynthetic processes necessary for axis growth.

Although protein is the major reserve material in soybean seeds, carbohydrates are an important source of respiratory substrates for energy production during germination and early seedling growth (Pazur et al., 1962; Wahab and Burris, 1975). The majority of the carbohydrates in soybeans are stored in the cotyledons and constitute approximately 12% of the dry weight of the mature seed. The soluble oligosaccharides stachyose and raffinose compose 35% of the carbohydrate content of the seed, with less than 1% starch (Abrahamson and Sudia, 1966; Adams et al., 1980; Yazdi-Samadi et al., 1977). However, sucrose is the most abdundant sugar, comprising nearly 65% of the carbohydrate fraction in the seed. Excised soybean axes were utilized in the following experiments to establish the importance of sucrose availability on axis growth, respiratory activity, and phosphorylated adenylate energy levels, and how these factors might be related to vigor deterioration exclusive of a carbohydrate supply from the cotyledons.

Materials and Methods

All experiments were conducted with soybean seeds which originated from the seed lot identified in Chapter II. The method for accelerated aging was the same for all experiments and is described in Chapter II. If necessary, accelerated aged seeds or excised axes from these seeds

were stored at 10° C and 45% relative humidity but for no longer than 10 days before use in any experiment. Nonaged seeds or excised axes were maintained under similar storage conditions.

Experiment 1. Effect of Externally Supplied Sucrose on the Growth of Axes Excised from Accelerated Aged and Nonaged Soybean Seeds

Embryonic axes were dissected from aged and nonaged seeds prior to imbibition. Growth tests were conducted using only excised axes 4 mm in length with attached plumules. Aseptic conditions were maintained throughout the experiment. Previous work revealed that the excised axes were essentially free of microbial contaminents. Therefore, the excised axes were not surface disinfected. For growth determinations, 4 replicates of 5 axes per treatment were placed in 10 cm diameter Petri dishes on 1.5% (weight/volume) agar (pH 6.1). To determine the effect of externally supplied sucrose on the growth of excised axes from aged and nonaged seeds, the media contained either 0%, 1%, 5%, or 10% (weight/volume) sucrose. The excised axes were all oriented in the same direction in the dishes with the abaxial surfaces in contact with the medium. The dishes were covered with lids, sealed with parafilm, and placed upright in a 25° C incubator, so that all radicle tips pointed downward. Growth tests with excised axes were conducted for 96 hours in darkness. Axis, hypocotyl, and root length measurements were taken at 24 hour intervals during this time without opening the dishes. Data were recorded only on axes where no microbial contamination could be observed. At 96 hours, axis fresh weights were determined and axis dry weights recorded after oven-drying at 70° C for 48 hours.

Experiment 2. Changes in Respiratory Activity During the Growth of Excised Axes of Soybean Seeds in Relation to Accelerated Aging and External Sucrose Availability

Following similar procedures described in the previous experiment replicated samples of 5 axes were dissected from aged and nonaged seeds and grown for up to 96 hours at 25° C on agar media containing or lacking 1% sucrose.

To evaluate the effect of sucrose availability on the respiratory activity of aged and nonaged axes, oxygen uptake was measured at 24 hour intervals on a Gilson single valve differential respirometer (Umbreit et al., 1959). Excised axis samples were placed into individual 15 ml Warburg respirometer flasks after axis, hypocotyl, and root lengths were measured. Three milliliters of distilled water were added to the main chamber of the flasks containing the axes grown in the absence of sucrose. The same volume of a 1% sucrose solution was included in the flasks containing the axes grown on the sucrose media. The center wells of all flasks contained a filter paper wick and 0.4 ml of a 10% KOH solution to absorb CO_2. The flasks were secured to the respirometer and continuously agitated at 100 cycles per minute in a 25° C water bath. After a 20 minute temperature equilibration period, the manometer valves were closed. The quantity of oxygen consumed per flask was read directly in µl after 30 minutes.

Initial axis respiratory activities (time 0) were determined by the amount of oxygen consumed during the latter half of the first hour of imbibition. Two flasks, prepared similarly as those for axes, but without axis material added, were included in each determination. These

flasks served as thermobarometers to correct for pressure changes due to temperature fluctuations.

After removal from the flasks and blotting with tissue paper, axis fresh weights were recorded. Axis dry weights were recorded after oven-drying at 70° C for 48 hours. Oxygen uptake of the excised axes was calculated as μl per hour per mg dry weight.

Experiment 3. Changed in Phosphorylated Adenylates and Energy Charge During the Growth of Excised Axes from Accelerated Aged and Nonaged Soybean Seeds

Replicated samples of 10 axes were dissected from aged and nonaged seeds and grown for 24, 48, 72, and 96 hours on agar media containing 1% sucrose according to the procedures described in the first experiment of this chapter. At each 24 hour growth period samples were taken for measurement of phosphorylated adenylate (ATP, ADP, AMP) concentrations from which energy charge values were determined. Prior to extraction, axis, hypocotyl, and root lengths of each sample were measured.

To extract the phosphorylated adenylates, axes were removed from the agar media and placed directly into test tubes containing 10 ml of boiling distilled water. The extract solutions were kept at 100° C in a boiling water bath for 10 minutes. Glass marbles were placed on the tops of the test tubes to minimize evaporation. After approximately 5 minutes of boiling, the axis tissues were ground by hand in the water bath using a teflon grinding bit to aid in extraction.

Immediately after boiling, sample extracts were cooled and kept on ice. A 10 ml volume of buffer containing 0.1 M HEPES and 0.1 M magnesium acetate, pH 7.5, was added to each extract solution. The solutions were centrifuged at 20,000 x g for 10 minutes at 4° C. Concentrations of ATP, ADP, and AMP in the axis extract solutions and axis

energy charge values were determined according to the procedure of Cohn and Obendorf (1976) described in Chapter IV. Adenosine phosphate concentrations were expressed as nmoles per axis from which energy charge values were then calculated.

Results and Discussion

Experiment 1. Effects of Externally Supplied Sucrose on the Growth of Axes Excised from Accelerated Aged and Nonaged Soybean Seeds

After the first 24 hours of growth, nonaged axes had elongated to a greater extent than aged axes regardless of external sucrose availability (Table V-1). A slightly greater increase in axis length was observed for nonaged axes grown on the 5% sucrose medium compared to nonaged axes grown on 1% or 0% sucrose. However, the lengths of nonaged axes grown on 10% sucrose were shorter than all other nonaged axes at 24 hours. The lengths of aged axes grown on the various sucrose media were the same at 24 hours with the exception of those grown on the 10% sucrose medium, where reduced elongation was noted. Although aged and nonaged axes continued to elongate during the following 24 hour growth period, a similar relationship between axis lengths and sucrose concentrations in the media was still evident. The reduced lengths of both aged and nonaged axes developing on the medium containing the highest carbohydrate concentration suggested that the sucrose may have osmotically limited elongation.

At 48 and 72 hours, nonaged axes grown on 5% sucrose were considerably longer than the other nonaged axes. However, the lengths of nonaged axes grown on the 1% sucrose medium were comparable to those of nonaged axes grown on the 5% medium by 96 hours.

Table V-1. Axis lengths of axes excised from accelerated aged and nonaged soybean seeds and grown on media differing in sucrose content.

Seed Treatment	Media Composition (percent sucrose)	Growth Time (hours)				
		0	24	48	72	96
		Axis Length (mm/axis)				
Nonaged	0	4.0a[z]	11.4b	16.9b	23.6b	29.9c
	1	4.0a	11.3b	16.7b	26.6b	36.4ab
	5	4.0a	12.0a	22.2a	35.5a	40.5a
	10	4.0a	9.9c	14.4c	23.3b	31.6bc
Aged	0	4.0a	8.1d	12.7d	15.6c	18.6d
	1	4.0a	8.2d	12.4d	14.7c	17.6d
	5	4.0a	8.5d	11.6d	13.2c	14.4d
	10	4.0a	7.5e	9.5e	11.7c	13.1d

[z]Mean separation in columns by Duncan's multiple range test, 5% level.

At no time during the 96 hour growth period did aged axis lengths equal those of nonaged axes. Although statistically significant differences were not found, there was a trend toward restricted elongation of aged axes as the carbohydrate concentration in the media increased.

Root length measurements presented in Table V-2 show that initiation of aged axis root growth lagged 24 hours behind that of nonaged axes, independent of external sucrose availability. Moreover, the rate of aged axis root growth was substantially slower compared to nonaged axes during the final 24 hour growth period. The differences in root growth between nonaged and aged excised axes may have been related to both delayed initiation and reduced activity of cell division in the aged axis root tips similar to that previously observed in the intact seedlings of aged seeds (Chapter IV).

Sucrose concentrations of the media had no affect on the root growth of aged axes. This finding suggests that reduced availability of this energy source was not a primary factor responsible for the impaired growth of excised axes following accelerated aging. In contrast to the aged axes, root growth of nonaged axes appeared to be influenced by external sucrose availability. Greater root growth occurred in these axes as the sucrose concentration in the media was increased up to 5%.

As opposed to the detrimental effect on root growth, accelerated aging did not result in impairment of hypocotyl growth of the excised axes past the first 24 hour growth period (Table V-3). This observation may be considered of primary importance, as it demonstrated that not all tissues within the axis were adversely effected to the same degree by accelerated aging. Both aged and nonaged axes generally exhibited

Table V-2. Root lengths of axes excised from accelerated aged and nonaged soybean seeds and grown on media differing in sucrose content.

Seed Treatment	Media Composition (percent sucrose)	Growth Time (hours)			
		24	48	72	96
		Root Length (mm/axis)			
Nonaged	0	0a[z]	4.9c	8.9c	12.3c
	1	0a	4.9b	13.2b	21.4b
	5	0a	10.0a	22.0a	26.4a
	10	0a	4.4b	12.8b	20.5b
Aged	0	0a	0c	1.8d	3.3d
	1	0a	0c	1.3d	3.2d
	5	0a	0c	1.0d	2.0d
	10	0a	0c	0.9d	1.5d

[z]Mean separation in columns by Duncan's multiple range test, 5% level.

Table V-3. Hypocotyl lengths of axis excised from accelerated aged and nonaged soybean seeds and grown on media differing in sucrose content.

Seed Treatment	Media Composition (percent sucrose)	Growth Time (hours)				
		0	24	48	72	96
		Hypocotyl Length (mm/axis)				
Nonaged	0	4.0a[z]	11.4b	12.0a	14.7a	17.6a
	1	4.0a	11.3b	11.8a	13.4b	15.0b
	5	4.0a	12.0a	12.2a	13.5b	14.1b
	10	4.0a	9.9c	10.0b	10.5d	11.1c
Aged	0	4.0a	8.1d	12.7a	13.8ab	15.3b
	1	4.0a	8.2d	12.4a	13.4b	14.4b
	5	4.0a	8.5d	11.6a	12.2c	12.4c
	10	4.0a	7.5d	9.5b	10.8d	11.6c

[z]Mean separation in columns by Duncan's multiple range test, 5% level.

shorter hypocotyl lengths as the concentration of sucrose in the media increased.

At each sucrose concentration nonaged axes had greater fresh weights than aged axes after 96 hours of growth (Table V-4). Although the dry weights of both aged and nonaged axes significantly increased from 0% to 1% sucrose concentration, there was a decrease in axis fresh weights at sucrose concentrations above 1%, possibly denoting an osmotic effect of the higher sucrose concentrations (5% and 10%). Thus, 0% or 1% concentrations of sucrose in the media were choosen for the experiments which follow. Maintaining sucrose concentrations in the media at 1% or less also minimized the risk of microbial contamination.

Experiment 2. Changes in Respiratory Activity During the Growth of Excised Axes of Soybean Seeds in Relation to Accelerated Aging and External Sucrose Availability

Impaired respiratory activity was observed in axes excised from aged soybean seeds soon after the onset of imbibition based on the rate of oxygen consumption (Table V-5). This observation is consistent with the findings of Leopold and Musgrave (1980), who reported a similar reduction in axis respiration of accelerated aged soybean seeds during the first hour of imbibition. They attributed the lower rate of respiration primarily to deterioration of the cytochrome pathway in the aged axes. Woodstock and Taylorson (1981) found greater quantities of ethanol and acetaldehyde in imbibed axes of soybean seeds after accelerated aging. From their results, they suggested that an imbalance between the activities of the glycolytic pathway and the tricarboxylic acid cycle may have been responsible for the impaired respiratory activity of the aged axes.

Table V-4. Fresh and dry weights of axes excised from accelerated aged and nonaged soybean seeds and grown for 96 hours on media differing in sucrose content.

Seed Treatment	Media Composition (percent sucrose)	Axis Fresh Weight (mg/axis)	Axis Dry Weight (mg/axis)
Nonaged	0	45.9a[z]	3.6cd
	1	45.2a	3.9ab
	5	34.8c	3.9ab
	10	24.9e	3.7bc
Aged	0	40.8b	3.5d
	1	38.6b	3.9a
	5	29.2d	3.7bc
	10	19.8f	3.5d

[z]Mean separation in columns by Duncan's multiple range test, 5% level.

Table V-5. Effect of externally supplied sucrose on respiratory activity (oxygen consumption) and growth characteristics of axes excised from unimbibed accelerated aged and nonaged soybean seeds.

Seed Treatment	Media Composition (percent sucrose)	Growth Time (hours) 0	24	48	72	96
		Axis Oxygen Consumption (μl/mg dry weight)				
Nonaged	0	0.7a[z]	6.2a	4.9b	3.7b	2.9b
	1	0.7a	5.6b	5.6b	4.6a	4.1a
Aged	0	0.3b	3.6c	5.0b	3.5b	2.6b
	1	0.3b	3.0d	4.8b	4.5a	3.8a
		Axis Fresh Weight (mg/axis)				
Nonaged	0	11.0a	24.2a	33.6a	40.3a	48.8a
	1	11.2a	24.4a	31.1a	39.7a	52.6a
Aged	0	10.1b	15.4b	23.6b	35.4b	36.3b
	1	9.9b	16.2b	26.0b	31.0c	40.6b
		Axis Dry Weight (mg/axis)				
Nonaged	0	4.2a	4.0a	4.1a	3.6b	3.9b
	1	4.5a	4.2a	4.2a	3.9a	4.3a
Aged	0	4.2a	3.8a	4.0a	3.6b	3.4c
	1	4.0a	4.0a	3.9a	3.7ab	3.8bc
		Axis Length (mm/axis				
Nonaged	0	4.0a	12.0a	16.2a	23.1a	28.8b
	1	4.0a	12.6a	16.6a	24.0a	36.6a
Aged	0	4.0a	7.8b	12.4b	15.8b	17.3c
	1	4.0a	7.8b	11.4b	14.6b	17.7c
		Root Length (mm/axis)				
Nonaged	0	0a	0a	3.6b	6.4b	8.8b
	1	0a	0a	5.0a	12.1a	20.9a
Aged	0	0a	0a	0a	0.2d	1.5c
	1	0a	0a	0c	1.5c	1.8c
		Hypocotyl Length (mm/axis)				
Nonaged	0	4.0a	12.0a	12.6a	16.7a	19.5a
	1	4.0a	12.0a	11.6a	11.9b	15.7b
Aged	0	0a	7.8b	12.4a	15.6a	15.8b
	1	0a	7.8b	11.4a	13.1b	15.9b

[z]Mean separation in columns by Duncan's multiple range test, 5% level.

The respiratory activity of the aged axes increased during the first 24 hour growth period, but the rate of oxygen consumption was still well below that of the axes excised from nonaged seeds. The lower respiration rate of the aged axes was evident whether or not the growth medium contained sucrose. This indicated that the reduced respiratory activity of the aged axes was not related to external sucrose availability, but rather an inability to utilize endogenous respiratory substrates at this time.

At 24 hours, nonaged axes grown on the sucrose medium had a lower respiration rate than nonaged axes grown on the medium without sucrose. A similar relationship between the respiratory rates and the sucrose content in the media was evident for aged axes as well. The reduction in the respiratory rates of axes grown on the sucrose medium is difficult to explain, as it did not appear to the be result of an osmotic affect of the sucrose limiting imbibition and rehydration of the tissues during this period.

The respiratory rates of the aged axes continued to increase up to 48 hours of growth independent of external sucrose availability. This indicated that respiratory substrates in the aged axes may still have been at levels adequate to meet their demand. In contrast to the aged axes, the respiratory rate of nonaged axes grown in the absence of sucrose declined during the 24 to 48 hour growth period, possibly due to the depletion of endogenous respiratory substrates. Supporting this contention was the fact that a higher level of respiratory activity was maintained in the nonaged axes when sucrose was included in the growth medium.

The respiratory activity of all axes declined during the latter 48 hours of growth, but to a lesser extent if sucrose was present in the growth media. This suggested that sucrose from the medium may have possibly been absorbed and utilized as a respiratory substrate by both the aged and nonaged axes. In the nonaged axes, the elevated rate of respiration was associated with improved axis growth as evidenced by greater root lengths and axis dry weights by 96 hours (Table V-5). More importantly, the increase in the respiratory activity of aged axes grown in the presence of sucrose had no beneficial effect on any axis growth parameter. Thus, it may be concluded that the detrimental effects of accelerated aging on excised axis growth cannot be overcome merely by an increase in respiratory activity.

Experiment 3. Changes in Phosphorylated Adenylates and Energy Charge During the Growth of Excised Axes from Accelerated Aged and Nonaged Soybean Seeds

There was no difference between the energy charge values of axes excised from aged and nonaged seeds prior to imbibition (Figure V-1). Energy charge values were low but within a range previously found to be typical of unimbibed axes of soybeans (Anderson, 1977; Rodaway et al., 1979) and other seeds such as pea (Brown, 1962) and pine (Ching and Ching, 1972). The low initial energy charge values were due to the predominance of AMP in the axes relative to the concentrations of ATP and ADP (Figures V-2, 3, 4). Axes excised from aged seeds had a higher concentration of total phosphorylated adenylates (ATP + ADP + AMP) than axes excised from nonaged seeds before imbibition had been initiated (Figure V-5). This suggested that biochemical mechanisms responsible for AMP synthesis might have been activated as the moisture content and

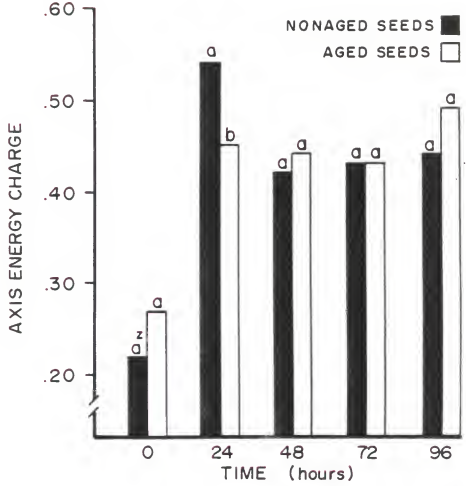

Figure V-1. Axis energy charge values of axes excised from nonaged and accelerated aged soybean seeds and grown on 1.5% agar media containing 1% sucrose.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

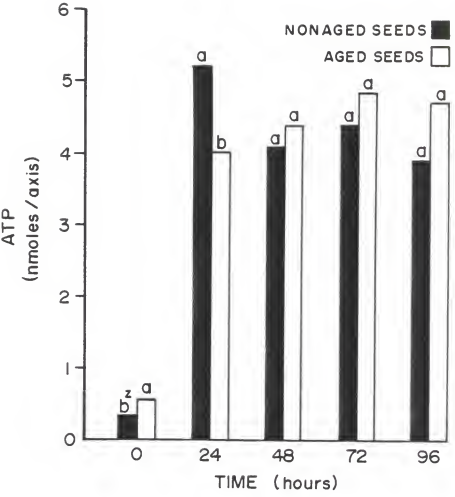

Figure V-2. Adenosine triphosphate content of axes excised from nonaged and accelerated aged soybean seeds and grown on 1.5% agar media containing 1% sucrose.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

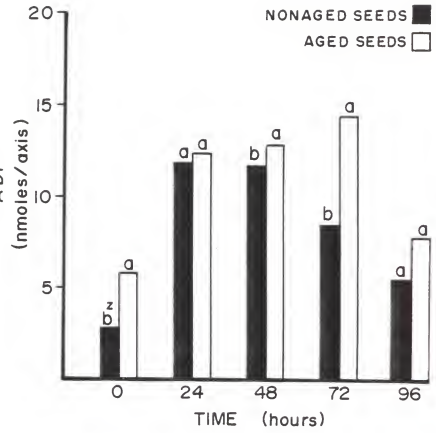

Figure V-3. Adenosine diphosphate content of axes excised from nonaged and accelerated aged soybean seeds and grown on 1.5% agar media containing 1% sucrose.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

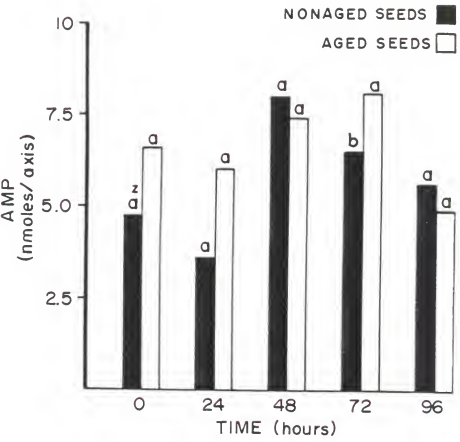

Figure V-4. Adenosine monophosphate content of axes excised from nonaged and accelerated aged soybean seeds and grown on 1.5% agar media containing 1% sucrose.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

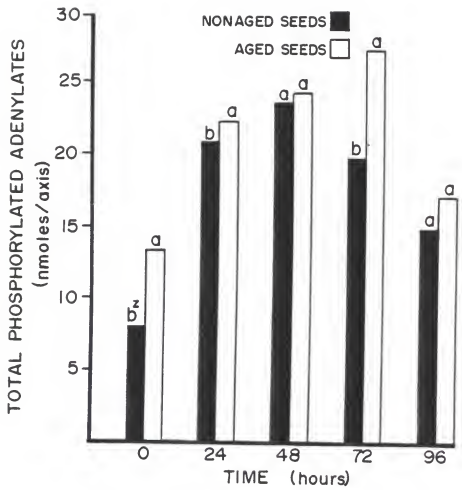

Figure V-5. Total phosphorylated adenylate content of axes excised from nonaged and accelerated aged soybean seeds and grown on 1.5% agar media containing 1% sucrose.

[z]Mean separation between treatments at each time by Duncan's multiple range test, 5% level.

temperature of the axes increased during the accelerated aging treatment.

Although the ATP concentration markedly increased in the aged axes, a greater quantity of ATP was detected in the nonaged axes after the first 24 hours of development on the 1% sucrose medium (Figure V-2). Consequently, aged axes had a lower energy charge value than nonaged axes at 24 hours (Figure V-1). According to the concept of energy charge (Atkinson, 1969), biosynthetic processes were more active in the nonaged axes than in the aged axes during the initial hours of axis hydration. This may have been a possible explanation for the greater length of the nonaged axes compared to the aged axes at 24 hours (Table V-6). Anderson (1977) reported that relatively lower ATP concentrations and energy charge values of aged soybean axes imbibed for 3 hours directly correlated to reduced protein and RNA synthesis in the axes as well as a decline in germinability. However, data were not presented regarding the subsequent axis growth of the deteriorated seeds used in his study.

During the 48 to 96 hour period of development, nonaged axes increased in length to a much greater extent than aged axes (Table V-6). As was observed in the previous 2 experiments of this chapter, the greater lengths of the nonaged axes were mainly due to their earlier initiation and faster rate of root growth compared to the aged axes. While accelerated aging resulted in a pronounced reduction in the rate of excised axis growth from 48 to 96 hours, neither the energy charge values nor the concentrations of ATP differed between aged and nonaged axes during this time (Figures V-1, 2). Thus, energy charge values and ATP concentrations appeared to be unrelated to the differences in

Table V-6. Growth measurements of axes excised from nonaged and accelerated aged soybean seeds used in phosphorylated adenylate content and energy charge determinations.

Seed Treatment	Germination Time (hours)				
	0	24	48	72	96
			Axis Length (mm/axis)		
Nonaged	4.0	11.4	18.4	27.3	36.4
Aged	4.0	8.4	11.9	13.7	17.2
	ns	*	*	*	*
			Root Length (mm/axis)		
Nonaged	0	0	5.6	13.6	20.4
Aged	0	0	0	0.7	2.5
	ns	ns	*	*	*
			Hypocotyl Length (mm/axis)		
Nonaged	4.0	11.4	12.8	13.7	16.0
Aged	4.0	8.4	11.9	13.0	14.7
	ns	*	ns	ns	ns

[z]Mean separation in columns by Duncan's multiple range test: ns = nonsignificant; * = significant at 5% level.

excised axis growth due to accelerated aging, regardless of equivalent external sucrose availability. These findings are in agreement with those reported in Chapter IV, where energy charge evaluations were determined from axes of intact aged and nonaged soybean seedlings. As discussed in Chapter IV, factors such as the rate of ATP turnover or its utilization efficiency may possibly have been of more importance in determining the role phosphorylated adenylates play in the regulation of axis growth.

Summary

Experiments were conducted with axes excised from accelerated aged and nonaged soybean seeds to establish the importance of sucrose availability on axis growth, respiratory activity, and phosphorylated adenylate levels, and how these factors may have been related to vigor deterioration independent of a carbohydrate supply from the cotyledons.

Although external sucrose concentrations up to 5% were associated with greater nonaged axis lengths, due primarily to increased root growth, external sucrose availability had no affect on aged axis growth. However, it was observed that accelerated aging had no detrimental influence on the capacity of the excised axes to increase hypocotyl lengths but delayed and severely inhibited root growth. In excised axes of both aged and nonaged seeds, sucrose concentrations above 1% limited fresh weight accumulation, possibly through an osmotic effect of the carbohydrate content of the media.

External sucrose availability improved the respiratory activity of excised aged and nonaged axes during the latter half of the 96 hour growth period. Only in the nonaged axes was the elevated respiration rate associated with an improvement in excised axis growth. It was

concluded that the detrimental effects of accelerated aging on excised axis growth could not be overcome merely by an increase in respiratory activity.

Energy charge evaluations and ATP concentrations generally appeared to be unrelated to the differences in excised axis growth that were attributed to accelerated aging.

The technique for growing excised seed axes employed in these experiments may be worthy of additional studies concerning the metabolic basis of seed vigor deterioration. The method offers a potential means for the controlled introduction of biochemical compounds or mineral elements into the growing axis which may modify or play an essential role in metabolic processes related to axis development. One approach may be to delve more specifically into various aspects of protein and nucleic acid synthesis in the developing axis. This could be accomplished by including specific radiolabelled precursors into the growth media followed by examination of not only the quantities but also the particular types of compounds synthesized. In addition, studies involving the incorporation of different concentrations and combinations of growth regulators into the media may be beneficial in discerning their role in deteriorated axis development.

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BIOGRAPHICAL SKETCH

David Floren James was born in Fremont, Ohio, on January 23, 1953. In June, 1971, he was graduated from Huron High School, Huron, Ohio. He enrolled at The Ohio State University in September, 1971, from which he received his Bachelor of Science in Agriculture degree with a major in plant pathology in June, 1975. He was awarded his Master of Science degree with a major in horticulture from The Ohio State University in June, 1977. During his graduate studies at The Ohio State University, the author served as a research associate for the Ohio Agricultural Research and Development Center, Wooster, Ohio. In September, 1977, he entered the University of Florida as a graduate research assistant for the Vegetable Crops Department to pursue his Doctor of Philosophy degree.

The author is a member of the American Society of Plant Physiologists and the American Society for Horticultural Science.

David was married to the former Holly Ann Baumring in October, 1981.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Daniel J. Cantliffe, Chairman
Professor of Vegetable Crops

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Chesley B. Hall
Professor of Vegetable Crops

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Thomas E. Humphreys
Professor of Botany

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Terril A. Nell
Assistant Professor of Ornamental Horticulture

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

S H West

Sherlie H. West
Professor of Agronomy

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1982

Jack L. Fry

Dean, College of Agriculture

Dean for Graduate Studies and Research